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Original article

# Halogenated flavanones as potential apoptosis-inducing agents: Synthesis and biological activity evaluation

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ABSTRACT

A series of halogenated flavanones were synthesized from 2-hydroxychalcones and tested for their cytotoxicity against a panel of human cancer cell lines. Among the synthesized compounds, 3′,7-dichloroflavanone (**2d**) showed the highest activity against MCF-7, LNCaP, PC3, Hep-G2, KB and SK-N-MC cells. However, 3′,6-dichloroflavanone (**2g**) with $IC_{50}$ value of 2.9 ± 0.9 μM was the most potent compound against MDA-MB-231 cells, being approximately 12 times more active than etoposide as reference drug. According to the flow-cytometric analysis, compound **2g** can induce apoptosis by 66.19 and 21.37% in PC3 and MDA-MB-231 cells, respectively. The results of acridine orange/ethidium bromide staining and TUNEL assay suggested that the cytotoxic activity of this compound in PC3 and MDA-MB-231 cells occurs via apoptosis.

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## 1. Introduction

Despite significant investment in the field of cancer chemotherapy, limited improvement in patient survival has been achieved in many countries. Cancer is the second leading cause of death worldwide after heart diseases [1,2]. Natural or semi-synthetic compounds may be used to prevent or treat the development of invasive cancers [2]. Flavonoids are naturally occurring polyphenolic compounds that have been reported to possess anticancer or anti-carcinogenic/antimutagenic activities [3]. These compounds possess a common phenylbenzopyrone structure (C6–C3–C6), consisting of two aromatic rings linked by three carbons that are usually in an oxygenated central pyran ring. They are categorized according to the saturation level and opening of the central pyran ring, mainly into flavones, isoflavones, flavonols, flavanonols, flavanols and flavanones (Fig. 1) [1,4,5]. Several beneficial biological effects of flavonoids including antioxidant, antitumor, and anti-inflammation properties have been ascertained in several previous studies [6–8]. Flavonoids have been identified to inhibit proliferation in many kinds of cultured human cancer cell lines, whereas less or no toxic to human normal cells [9–11]. The significant anticancer properties of flavonoids may be via induction of apoptosis [5].

Flavanones have been a potential source in the search for new lead compounds in the field of cancer chemotherapy. Usman et al. [12] had reported cytotoxic properties of flavanones isolated from the tree barks of *Cryptocarya costata*. A study of eight flavanones on colorectal carcinoma cells indicated that 2′-OH flavanone showed the most potent cytotoxic effect on these cancer cells, and cell death induced by 2′-OH flavanone was via the occurrence of DNA ladders, apoptotic bodies, and hypodiploid cells, all characteristics of apoptosis [13]. In 2007, Hsiao et al. described that flavanone and 2′-OH flavanone inhibited cell growth of A549, LLC, AGS, SK-Hepl and HA22T cancer cells, while other flavanones (4′-OH flavanone, 6-OH flavanone, naringin and naringenin) showed little or no inhibition [14]. The results of another study explored that synthetic flavanone derivatives have strong anti-proliferative effects on human breast cancer cells by way of p53-mediated apoptosis and the induction of cell cycle arrest at the G1 phase [15]. Also, Choi et al. reported that 4′,7-dimethoxyflavanone, exhibits potent anticancer activity and induces cell cycle arrest and apoptosis in human breast cancer MCF-7 cells [16].

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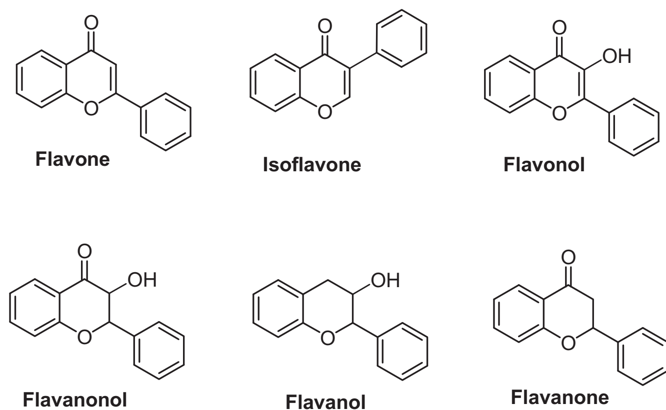



**Fig. 1.** The main structural categories of flavonoids.

It is now well documented that most cytotoxic anticancer agents induce apoptosis. Therefore, an attractive method for cancer chemoprevention or chemotherapy is new therapeutic agents that induce tumor cells death [17–19]. Because no systemic inflammatory responses have been observed in cells induced to undergo apoptosis, several studies have suggested that the induction of cell death via apoptosis reserves a physiological advantage in the cancer treatment [20,21].

In continuation of our previous work on the synthesis of potential cytotoxic agents and apoptosis inducers [22–25], and based on the diverse biological activities of flavanone derivatives, in this paper we have synthesized and evaluated cytotoxicity and apoptosis-inducing activity of a series of halogenated flavanones **2** in order to develop novel anticancer agents. These compounds are small molecules which consist of chromanone ring and aryl ring attached to the 2 position. Since halogens like chlorine, are very useful to modulate the electronic and steric characteristics of drugs and may also influence the hydrophilic–hydrophobic balance of the molecules, thus chlorine substitution on the chromanone ring and on the C-2 attached phenyl ring was used for structural modification and modulation of basic pharmacophore of flavanones.

## 2. Chemistry

A general synthesis of the flavanone derivatives **2a**–**k** is shown in Scheme 1. The condensation of 2-hydroxyacetophenone derivatives with the corresponding aldehyde in a basic media afforded hydroxychalcones **1a**–**k**. The compounds **1a**–**k** were cyclized in refluxing ethanol in the presence of sodium acetate to give flavanone derivatives **2a**–**k**. The structural characterization of flavanone compounds **2a**–**k** is based on their $^1$H NMR and IR spectral data. For example, their $^1$H NMR spectra showed typical chemical shifts and coupling pattern of the H-2, H-3ax and H-3eq protons of chroman ring in flavanone structures (Fig. 2). In the $^1$H NMR spectra of flavanones (Table 1), the H-2 signal appears at approximately at 5.24–5.88 ppm as doublet of doublet. The resonances of the diastereotopic H-3ax and H-3eq protons occurred approximately at 3.03–3.20 and 2.80–2.90 ppm as two doublets of doublets.

The H-3ax and H-3eq of chroman ring are coupled with a constant of 16.4–17.6 Hz related to the geminal coupling. The value of the coupling constant between H-2 and H-3ax is too large ($J_{2,3ax}$ = 12.4–13.6 Hz) which can only arise from a *trans*-diaxial coupling, thus H-2 is axial and the 2-aryl group has equatorial orientation. Furthermore, the value of $J_{2,3eq}$ which is in the range of 2.8–3.2 Hz confirms the conformation of the flavanone structure (Fig. 2). FT-IR spectra of flavanone derivatives also revealed a very strong band about 1642–1697 cm$^{-1}$ related to C=O stretching.

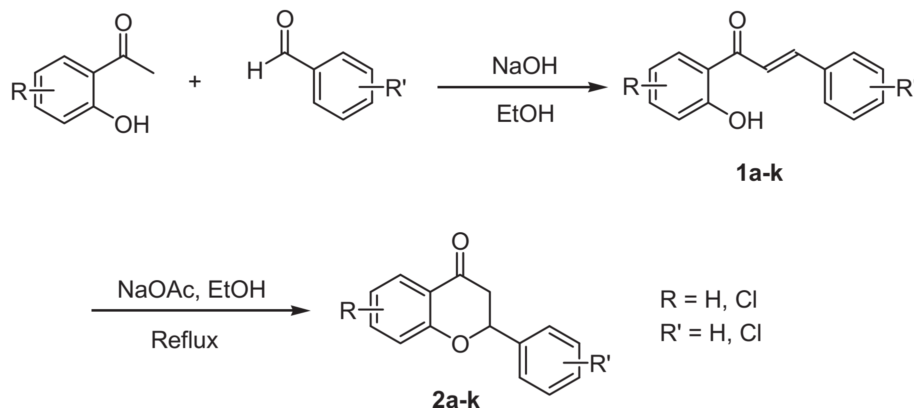
## 3. Biology

### 3.1. In vitro cytotoxicity assay

The in vitro cytotoxic activity of the test compounds **2a**–**k** against eight human cancer cell lines include MCF-7, MDA-MB-231 (human breast cancer), LNCaP, PC3 (human prostate cancer), Hep-G2 (human liver carcinoma), KB (human nasopharyngeal epidermoid carcinoma), SK-N-MC (human neuroblastoma) and K-562 (human erythroleukemic) was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay [26]. The results of cytotoxic assay in comparison with etoposide were listed in Table 2.

### 3.2. Acridine orange/ethidium bromide staining method

Apoptosis was determined morphologically after staining PC3 and MDA-MB-231 cells with acridine orange/ethidium bromide using fluorescence microscopy according to the previously described method [27]. Acridine orange penetrates into living and dead cells, emitting green fluorescence as a result of intercalation in double-stranded DNA. Ethidium bromide emits red fluorescence after intercalation in DNA of cells with an altered cell membrane. Ethidium bromide staining due to loss of membrane integrity identifies the population of late stage of apoptotic cells and necrotic cells. Analysis of the acridine orange/ethidium bromide staining of the synthetic compounds **2d** and **2g** are shown in Fig. 3.




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

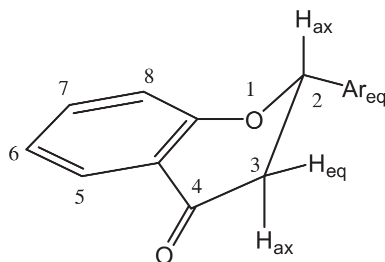



**Fig. 2.** Atom numbering and half-chair conformation of flavanone structure in which 2-aryl group exists predominantly in the equatorial orientation.

### *3.3. Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay*

For detection of early stages of apoptosis in PC3 and MDA-MB-231 cells, TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) analysis was used. Generation of free 3′-OH DNA fragments was determined using TUNEL analysis and quantified by flow cytometry. The results are shown in Fig. 4.

## 4. Results and discussion

All the synthesized compounds **2a**–**k** were evaluated for their cytotoxic effects on a panel of eight human tumor cell lines. For each compound, 50% inhibitory concentration ($IC_{50}$) was calculated and presented in Table 2.

Results of the MTT assay showed that flavanones with different chloro-substituent exhibited differential cytotoxicity in various human cancer cells lines. Although the unsubstituted flavanone **2a** showed marginal or no activity against all cell lines ($IC_{50}$ values $> 50$ μM) but chloro-substituted compounds exhibited significant activity against various cell lines with the exception of K-562 cells.

Among the tested compounds, compound **2d** with chlorine on 3′ and 7 positions showed the highest cytotoxic activity against MCF-7, LNCaP, PC3, Hep-G2, KB and SK-N-MC cells. Against MDA-MB-231 cell line, 3′,6-dichloroflavanone (**2g**) was the most potent compound with $IC_{50}$ value of $2.9 \pm 0.9$ μM. This compound was approximately 12 times more active than etoposide as reference drug. In the case of human neuroblastoma (SK-N-MC), compounds **2b** and **2c** followed by **2d** and **2e** (7-chloroflavanone derivatives **2b**–**e**) were the most active compounds being more potent than etoposide. The activity of compounds **2g**–**i** against SK-N-MC was comparable to that of etoposide. The $IC_{50}$ values of compounds against K-562 cell line revealed that flavanones with no substituent on chroman ring and possessing chlorine on phenyl group (compounds **2i**–**k**) showed good growth inhibitory activity at concentrations less than 25 μM. Among them, 3′-chloro-analog **2j** was the most potent compound against K-562 cell line.

The comparison of parent compound **2a** with chlorophenyl analogs **2i**–**k** demonstrated that the introduction of a chlorine atom on the 2-phenyl ring of flavanone increases the tumor growth inhibitory effects of flavanone against all cell lines. The cytotoxic activity of 7-chloro compound **2b** was more than that of parent compound **2a**. Thus, the presence of chloro group on chroman ring was favorable for cytotoxic activity. Although, chloro substituent on chroman ring or 2-phenyl group individually could increase the activity, but the combination pattern of substituents had a different effect on various cell lines. However, the highest activity was observed with 3′-chloro analogs (**2d** against MCF-7, LNCaP, PC3, Hep-G2, KB and SK-N-MC, **2g** against MDA-MB-231 and **2j** against K-562).

Among the halogenated compounds described in this study, the cytotoxic activity of 4′-chloroflavanone (**2k**) against MCF-7 and MDA-MB-453 cells has been reported [15]. This compound was included in our study for comparing its activity with poly-chlorinated compounds and other regio-isomers. As seen from data, our compounds for example 3′,7-dichloroflavanone (**2d**) had a better profile of cytotoxic activity in comparison to that of 4′-chloroflavanone (**2k**).

The synthetic compounds **2d** and **2g** were used to identify apoptotic or necrotic cell death in MDA-MB-231 and PC3 cell lines. Since there is an essential need to develop drugs for 'triple-negative' breast cancer – a tumor subtype that is categorized as being aggressive and lacking effective targeted treatments, such as endocrine therapies and anti-HER2 strategies [28] and because of potent activity of compound **2g** against this kind of tumor cell line (MDA-MB-231), our laboratory used MDA-MB-231 and androgen-refractory and chemotherapy-resistant PC3 prostate cancer cell line, to elucidate the death type induced by synthetic compounds.

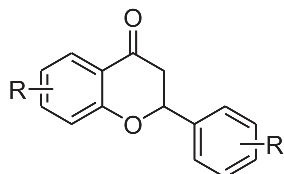
Analysis of the acridine orange/ethidium bromide staining revealed that the synthetic compounds **2d** and **2g** reduced cell viability and induced apoptosis in human prostate and breast cancer cell lines. The appearance of chromatin condensation, nuclear fragmentation and shift from early to late apoptosis are evident in Fig. 3. In this figure, viable cells are uniformly green, early apoptotic cells are green and contained chromatin condensation and nuclear fragmentation. Late apoptotic cells incorporate ethidium bromide and therefore stained orange and in contrast to necrotic cells, they show condense and often fragmented nuclei.

These observations were confirmed by the results obtained in the TUNEL assay, which detects early stages of apoptosis. We used TUNEL analysis as a quantitative method of determining apoptosis (Fig. 4(A) and (B)). Fig. 4(C) and (D) summarizes the data and shows a highly significant increase in the apoptotic index (as demonstrated by an increase in the fluorescein isothiocyanate (FITC)-positive fraction) in PC3 and MDA-MB-231 cells treated with synthetic compounds compared with negative control. The results also revealed that the percentage of PC3 cells undergoing apoptosis after 12 h of exposure to compounds **2d** and **2g** was higher than etoposide (49.48% and 66.19% apoptosis in the **2d**- and **2g**-treated cells, respectively versus 17.25% in etoposide-treated cells). Exposure of MDA-MB-231 cells to $IC_{50}$ concentrations of **2d** and **2g** induced apoptosis in 44.66% and 21.67% of the cells after 12 h,

**Table 1**
Selected $^{1}H$ NMR spectral data of compound **2a**–**k**.

| Compound | R | R′ | δ H-2 | δ H-3 | | $^{2}J_{3ax,3eq}$ | $^{3}J_{2ax,3eq}$ | $^{3}J_{2ax,3ax}$ |
|---|---|---|---|---|---|---|---|---|
| **2a** | H | H | 5.24 | 3.2 | 2.88 | 16.8 | 3.2 | 13.2 |
| **2b** | 7-Cl | H | 5.49 | 3.08 | 2.90 | 17.2 | 2.8 | 12.8 |
| **2c** | 7-Cl | 2′-Cl | 5.88 | 3.03 | 2.81 | 16.8 | 2.8 | 13.6 |
| **2d** | 7-Cl | 3′-Cl | 5.47 | 3.09 | 2.90 | 16.8 | 3.0 | 12.8 |
| **2e** | 7-Cl | 4′-Cl | 5.47 | 3.03 | 2.89 | 16.8 | 3.0 | 12.9 |
| **2f** | 6-Cl | 2′-Cl | 5.87 | 3.05 | 2.89 | 17.6 | 2.8 | 13.6 |
| **2g** | 6-Cl | 3′-Cl | 5.47 | 3.09 | 2.90 | 16.8 | 3.0 | 12.8 |
| **2h** | 6-Cl | 4′-Cl | 5.47 | 3.09 | 2.90 | 16.8 | 3.0 | 12.8 |
| **2i** | H | 2′-Cl | 5.88 | 3.04 | 2.89 | 16.8 | 2.8 | 13.2 |
| **2j** | H | 3′-Cl | 5.46 | 3.03 | 2.89 | 16.4 | 2.8 | 12.4 |
| **2k** | H | 4′-Cl | 5.47 | 3.04 | 2.88 | 16.8 | 2.8 | 12.8 |

**Table 2**
Cytotoxic activity ($IC_{50}$, μM)[a] of synthetic flavanone against a panel of cancer cell line after 48 h treatment.




| Compd | R | R′ | MCF-7 | MDA-MB-231 | LNCaP | PC3 | Hep-G2 | KB | SK-N-MC | K-562 |
|---|---|---|---|---|---|---|---|---|---|---|
| **2a** | H | H | >100 | 51.8 ± 2.4 | 94.6 ± 12.7 | 55.4 ± 7.1 | >100 | >100 | 77.5 ± 10.6 | >100 |
| **2b** | 7-Cl | H | 19.3 ± 6.0 | 8.2 ± 2.9 | 59.5 ± 4.9 | 17.7 ± 7.4 | 9.8 ± 1.5 | 25.2 ± 4.4 | 10.3 ± 2.3 | >100 |
| **2c** | 7-Cl | 2′-Cl | 20.7 ± 8.2 | 7.7 ± 1.8 | >100 | >100 | >100 | 47.3 ± 12.3 | 10.9 ± 1.1 | >100 |
| **2d** | 7-Cl | 3′-Cl | 11.3 ± 0.4 | 10.9 ± 2.5 | 35.9 ± 6.6 | 11.6 ± 4.1 | 5.7 ± 0.3 | 14.7 ± 0.2 | 12.8 ± 2.1 | 78.8 ± 9.5 |
| **2e** | 7-Cl | 4′-Cl | 13.0 ± 2.6 | 9.4 ± 1.3 | 58.1 ± 8.3 | 12.4 ± 4.5 | 21.9 ± 7.2 | >100 | 16.2 ± 1.6 | >100 |
| **2f** | 6-Cl | 2′-Cl | >100 | 36.2 ± 5.9 | 53.1 ± 4.1 | 27.4 ± 1.4 | >100 | >100 | >100 | >100 |
| **2g** | 6-Cl | 3′-Cl | 25.8 ± 2.1 | 2.9 ± 0.9 | 38.1 ± 4.4 | 12.2 ± 4.2 | 20.7 ± 3.8 | 76.9 ± 13.4 | 24.0 ± 0.7 | >100 |
| **2h** | 6-Cl | 4′-Cl | 14.5 ± 2.0 | 15.3 ± 5.5 | 55.7 ± 9.5 | 47.9 ± 7.5 | 13.7 ± 0.1 | >100 | 23.1 ± 4.6 | >100 |
| **2i** | H | 2′-Cl | 19.3 ± 6.2 | 20.3 ± 4.3 | 57.2 ± 4.2 | 43.8 ± 5.1 | 11.0 ± 1.5 | >100 | 24.0 ± 7.4 | 22.9 ± 0.1 |
| **2j** | H | 3′-Cl | 23.4 ± 9.7 | 38.3 ± 5.7 | 59.9 ± 10.6 | 51.3 ± 8.5 | 13.7 ± 0.1 | 26.9 ± 3.5 | 32.2 ± 2.8 | 18.6 ± 6.6 |
| **2k** | H | 4′-Cl | 22.9 ± 3.4 | 44.3 ± 3.9 | 56.0 ± 7.0 | 28.3 ± 7.7 | 32.5 ± 0.1 | 32.5 ± 5.0 | 38.2 ± 5.5 | 21.5 ± 2.7 |
| **Etoposide** | | | 31.3 ± 2.5 | 34.9 ± 4.2 | 16.8 ± 2.1 | 36.9 ± 4.7 | 19.2 ± 3.1 | 9.9 ± 2.8 | 23.9 ± 1.8 | 14.3 ± 2.8 |

[a] Data are mean ± SD of 3 independent experiments ($n = 3$).

respectively. These data suggested that the cytotoxic activity of **2d** and **2g** in PC3 and MDA-MB-231 cells occurs via apoptosis.

## 5. Conclusion

It has been demonstrated that the modulation of flavanone structure could increase antitumor activity. Thus, chlorine substitution on the chromanone ring and on the C-2 attached phenyl ring was used for structural modification and modulation of basic pharmacophore of flavanones. Therefore, a series of halogenated flavanones were synthesized from 2-hydroxychalcone derivatives and tested for their cytotoxicity against a panel of human cancer cell lines. Among the synthesized compounds, 3′,7-dichloroflavanone (**2d**) showed the better profile of cytotoxicity. However, 3′,6-dichloroflavanone (**2g**) with $IC_{50}$ value of 2.9 ± 0.9 μM was the most potent compound against MDA-MB-231 cells, being approximately 12 times more active than etoposide. According to the flow-cytometric analysis, compound **2g** can induce apoptosis by 66.19 and 21.37% in PC3 and MDA-MB-231 cells, respectively. The results of acridine orange/ethidium bromide staining and TUNEL assay suggested that the cytotoxic activity of this compound in PC3 and MDA-MB-231 cells occurs via apoptosis.

## 6. Experimental protocols

All chemicals and solvents used in this study were purchased from Merck chemical. Merck silica gel $F_{254}$ plates were used for analytical TLC. Melting points were determined on a Kofler hot stage apparatus and are uncorrected. The IR spectra were obtained on a Shimadzu 470 spectrophotometer (potassium bromide disks). The $^1$H NMR and $^{13}$C NMR spectra were recorded using Bruker 400 and Bruker 500 spectrometers, respectively. Chemical shifts are reported in parts per million (ppm) relative to TMS as internal standard.

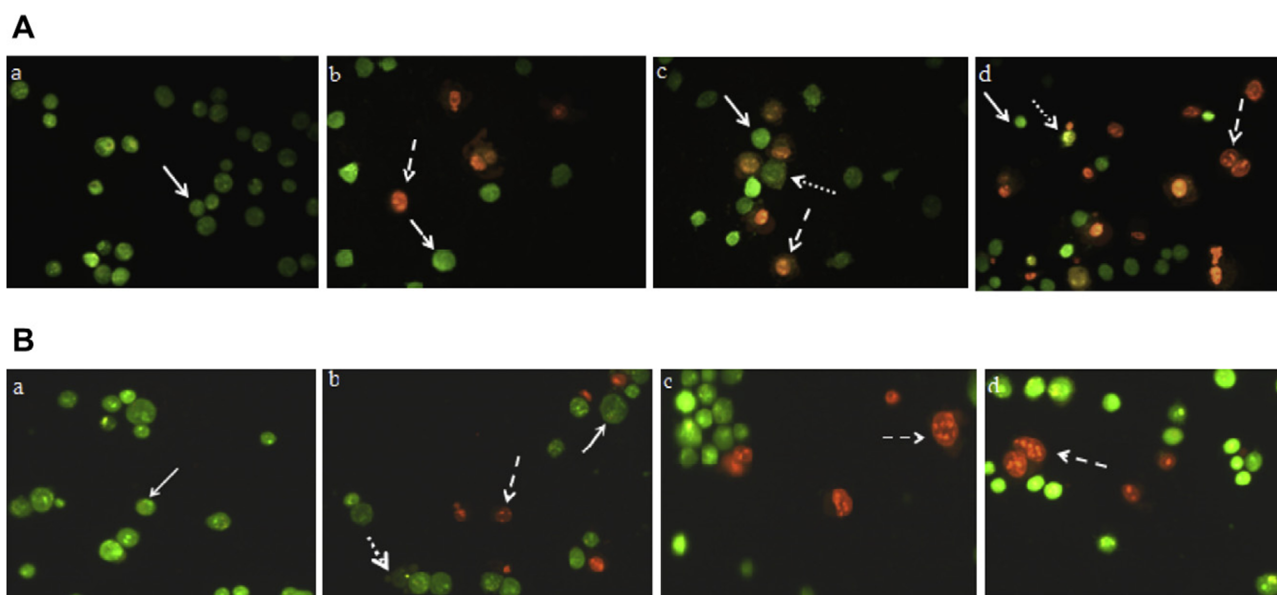



**Fig. 3.** Acridine orange/ethidium bromide double staining of PC3 (A) and MDA-MB-231 (B) cells with characteristic symptoms of apoptosis: a DMSO 1% as control, b, c and d, cells treated with $IC_{50}$ concentrations of etoposide, compounds **2d** and **2g**, respectively for 12 h. White arrow indicates live cells, dotted arrow shows early phase of apoptosis and dashed arrow indicates late phase of apoptosis. The images of cells were taken with a fluorescence microscope at 400×.

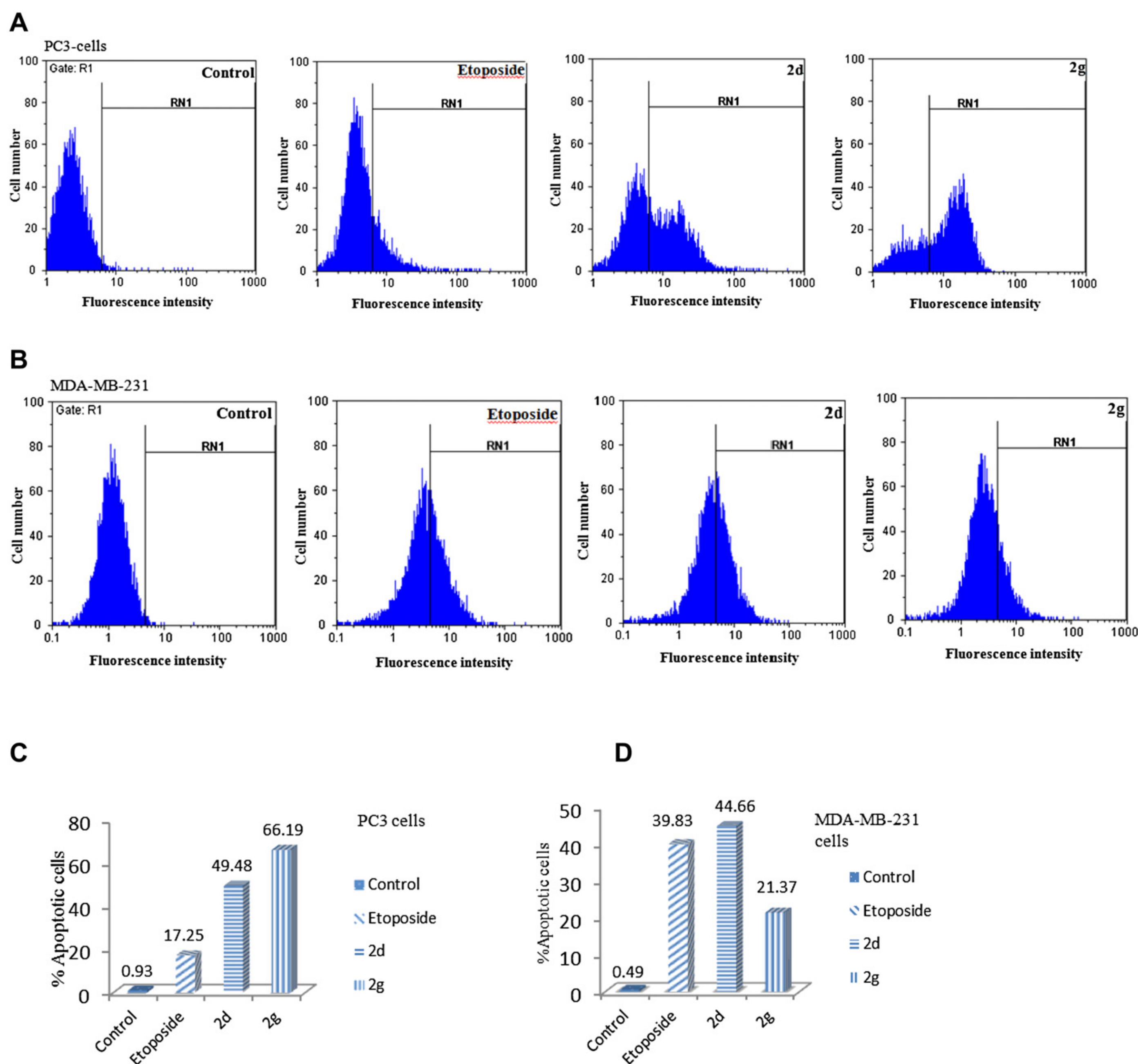



**Fig. 4.** Flow-cytometric analysis of PC3 and MDA-MB-231 cells. Generation of free 3′-OH DNA fragments was determined using TUNEL analysis and quantitated by flow cytometry. (A) PC3 cells treated with DMSO 1% (negative control) or with $IC_{50}$ values of etoposide (positive control) and compounds **2d** and **2g** for 12 h. (B) MDA-MB-231 cells treated with DMSO 1% (negative control) or with $IC_{50}$ values of etoposide (positive control) and **2d** and **2g** for 12 h. Percentage of FITC-positive events (% apoptotic cells) was calculated (C); and (D) % apoptotic cells of PC3 and MDA-MB-231 cell lines treated with DMSO 1% (negative control) or with $IC_{50}$ values of etoposide (positive control), compounds **2d** and **2g** respectively for 12 h.

### 6.1. General procedure for the synthesis of 2-hydroxychalcones **1**

To a well stirred solution of the aldehyde (10 mmol) and the appropriately substituted acetophenone (10 mmol) in methanol (30 ml), was added 50% NaOH aqueous solution (1 ml) and the reaction was stirred overnight at room temperature. The precipitated solid was filtered and recrystallized from hot methanol to give pure products. After completion of the reaction if the solid was not precipitated, the solution was neutralized with diluted HCl solution (1 N) and extracted with chloroform. The combined organic layers were dried ($MgSO_4$), filtered, and evaporated in vacuo. The residues were purified by column chromatography. Compounds **1a** and **1f–h**, were prepared according to the previously reported method [29].

#### 6.1.1. (E)-1-(4-chloro-2-hydroxyphenyl)-3-phenylprop-2-en-1-one (**1b**)

Yield 85%; m.p. 118–119 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 3300–3440 (OH), 1638 (C=O); $^{1}$H NMR (400 MHz, $CDCl_3$) $\delta$: 13.01 (s, 1H, OH), 7.94 (d, 1H alkene, $J$ = 16 Hz), 7.85 (d, 1H phenyl, $J$ = 8.4 Hz), 7.67–7.70 (m, 2H phenyl), 7.59 (d, 1H alkene, $J$ = 16 Hz), 7.41–7.46 (m, 3H, phenyl), 7.06 (s, 1H phenyl), 6.93 (d, 1H phenyl, $J$ = 8.4 Hz).

*6.1.2. (E)-1-(4-chloro-2-hydroxyphenyl)-3-(2-chlorophenyl)prop-2-en-1-one (**1c**)*

Yield 81%; m.p. 134–135 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 3300–3440 (OH), 1646 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 12.94 (s, 1H, OH), 8.33 (d, 1H alkene, $J$ = 15.6 Hz), 7.83 (d, 1H phenyl, $J$ = 8.8 Hz), 7.76 (d, 1H phenyl, $J$ = 7.2 Hz), 7.57 (d, 1H alkene, $J$ = 15.6 HZ), 7.48 (d, 1H phenyl, $J$ = 7.2 Hz), 7.32–7.40 (m, 2H phenyl), 7.06 (d, 1H phenyl, $J$ = 2.4 Hz), 6.93 (d, 1H phenyl, $J$ = 8.8 Hz).

*6.1.3. (E)-1-(4-chloro-2-hydroxyphenyl)-3-(3-chlorophenyl)prop-2-en-1-one (**1d**)*

Yield 76%; m.p. 124–125 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 3340–3461 (OH), 1642 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 12.92 (s, 1H, OH), 7.84–7.88 (m, 2H phenyl & alkene), 7.67 (s, 1H phenyl), 7.58 (d, 1H alkene, $J$ = 15.2 Hz), 7.50–7.54 (m, 2H phenyl), 7.40 (d, 1H phenyl, $J$ = 7.6 Hz), 7.06 (s, 1H phenyl), 6.95 (d, 1H phenyl, $J$ = 8.8 Hz).

*6.1.4. (E)-1-(4-chloro-2-hydroxyphenyl)-3-(4-chlorophenyl)prop-2-en-1-one (**1e**)*

Yield 79%; m.p. 129–130 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 3400–3421(OH), 1635 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 12.90 (s, 1H, OH), 7.88 (d, 1H alkene, $J$ = 16 Hz), 7.83 (d, 2H phenyl, $J$ = 8 Hz), 7.60 (d, 1H phenyl, $J$ = 8.8 Hz), 7.55 (d, 1H alkene, $J$ = 16 Hz), 7.42 (d, 2H phenyl, $J$ = 8 Hz), 7.05 (d, 1H phenyl, $J$ = 2 Hz), 6.93 (dd, 1H phenyl, $J$ = 8.8 and 2 Hz).

*6.1.5. (E)-3-(2-chlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**1i**)*

Yield 83%; m.p. 121–123 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 3428 (OH), 1637 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 12.73 (s, 1H, OH), 8.31 (d, 1H alkene, $J$ = 15.6 Hz), 7.91 (d, 1H phenyl, $J$ = 7.6 Hz), 7.77 (d, 1H phenyl, $J$ = 6.8 Hz), 7.63 (d, 1H alkene, $J$ = 15.6 Hz), 7.46–7.54 (m, 2H phenyl), 7.37–7.40 (m, 2H phenyl),7.05 (d, 1H phenyl, $J$ = 8.8 Hz), 6.93 (t, 1H phenyl, $J$ = 7.6 Hz).

*6.1.6. (E)-3-(3-chlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**1j**)*

Yield 87%; m.p. 101–103 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 3438 (OH), 1642 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 12.78 (s, 1H, OH), 7.92 (d, 1H phenyl, $J$ = 8.4 Hz), 7.85 (d, 1H alkene, $J$ = 16 Hz), 7.63–7.67 (m, 2H phenyl & alkene), 7.52 (t, 2H phenyl, $J$ = 6.8 Hz), 7.36–7.41 (m, 2H phenyl), 7.04 (d, 1H phenyl, $J$ = 8.4 Hz), 6.96 (t, 1H phenyl, $J$ = 7.8 Hz).

*6.1.7. (E)-3-(4-chlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**1k**)*

Yield 86%; m.p. 148–149 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 3432 (OH), 1631 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 12.78 (s, 1H, OH), 7.92 (d, 1H phenyl, $J$ = 8.4 Hz), 7.85 (d, 1H alkene, $J$ = 16 Hz), 7.63–7.67 (m, 2H phenyl & alkene), 7.52 (t, 2H phenyl, $J$ = 6.8 Hz), 7.36–7.41 (m, 2H phenyl), 7.04 (d, 1H phenyl, $J$ = 8.4 Hz), 6.96 (t, 1H phenyl, $J$ = 7.8 Hz).

### *6.2. General procedure for the synthesis of flavanones **2a–k***

To a well stirred solution of an appropriate chalcone (1 mmol) and sodium acetate (500 mg) in ethanol (5 ml), was added 3 drops of water and the mixture was refluxed overnight. The reaction mixture was poured into cold water and extracted with ethyl acetate. The organic phase was washed with saturated NaCl solution and dried ($Na_2SO_4$). After removing the solvent, the residue was purified by column chromatography using petroleum ether/ethyl acetate (9:1) as eluent to give compound **2a–k**.

*6.2.1. 2-Phenylchroman-4-one (**2a**)*

Yield 89%; m.p. 76–78 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 1696 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 7.93 (dd, 1H, $J$ = 1.2 and 8 Hz, $H_5$ chroman), 7.37–7.53 (m, 5H, phenyl), 7.02–7.09 (m, 3H, $H_6$, $H_7$ and $H_8$ chroman), 5.24 (dd, 1H, $J$ = 3.2 and 13.2 Hz, $H_2$ chroman), 3.2 (dd, 1H, $J$ = 13.2 and 16.8 Hz, $H_3$ chroman), 2.88 (dd, 1H, $J$ = 3.2 and 16.8 Hz, $H_3$ chroman). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$: 191.96, 161.53, 138.71, 136.18, 128.84, 128.76, 127.03, 126.13, 121.60, 120.91, 118.11, 79.58, 44.65. Anal. Calcd for $C_{15}H_{12}O_2$: C, 80.34; H, 5.39. Found: C, 79.98; H, 5.01.

*6.2.2. 7-Chloro-2-phenylchroman-4-one (**2b**)*

Yield 67%; m.p. 87–88 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 1676 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 7.86 (d, 1H, $J$ = 8.6 Hz, $H_5$ chroman), 7.39–7.46 (m, 5H, H phenyl), 7.08 (d, 1H, $J$ = 1.6 Hz, $H_8$ chroman), 7.03 (d, 1H, $J$ = 1.6 and 8.6 Hz, $H_6$ chroman), 5.49 (dd, 1H, $J$ = 2.8 and 12.8 Hz, $H_2$ chroman), 3.08 (dd, 1H, $J$ = 12.8 and 17.2 Hz, $H_3$ chroman), 2.90 (dd, 1H, $J$ = 2.8 and 17.2 Hz, $H_3$ chroman). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$: 190.39, 161.57, 142.16, 136.72, 134.79, 129.11, 128.31, 127.47, 122.64, 119.44, 118.27, 79.21, 44.26. Anal. Calcd for $C_{15}H_{11}ClO_2$: C, 69.64; H, 4.29. Found: C, 69.34; H, 4.31.

*6.2.3. 7-Chloro-2-(2-chlorophenyl)chroman-4-one (**2c**)*

Yield 65%; m.p. 62–64 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 1642 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 7.89 (d, 1H, $J$ = 8.2 Hz, $H_5$ chroman), 7.45 (d, 1H, $J$ = 7.6 Hz, $H_{3'}$), 7.38–7.43 (m, 2H, $H_{4'}$ and $H_{5'}$), 7.33 (d, 1H, $J$ = 7.2 Hz, $H_{6'}$), 7.10 (d, 1H, $J$ = 1.6 Hz, $H_8$ chroman), 7.06 (dd, 1H, $J$ = 1.6 and 8.2 Hz, $H_6$ chroman), 5.88 (dd, 1H, $J$ = 2.8 and 13.6 Hz, $H_2$ chroman), 3.03 (dd, 1H, $J$ = 2.8 and 16.8 Hz, $H_3$ chroman), 2.81 (dd, 1H, $J$ = 13.6 and 16.8 Hz, $H_3$ chroman). Anal. Calcd for $C_{15}H_{10}Cl_2O_2$: C, 61.46; H, 3.44. Found: C, 61.32; H, 3.32.

*6.2.4. 7-Chloro-2-(3-chlorophenyl)chroman-4-one (**2d**)*

Yield 58%; m.p. 93–94 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 1697 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 7.86 (d, 1H, $J$ = 8.8 Hz, $H_5$ chroman), 7.49 (s, 1H, $H_{2'}$), 7.37 (m, 1H, $H_{5'}$), 7.30–7.36 (m, 2H, $H_{4'}$ and $H_{6'}$), 7.10 (d, 1H, $J$ = 2.2 Hz, $H_8$ chroman), 7.05 (dd, 1H, $J$ = 2.2 and 8.8 Hz, $H_6$ chroman), 5.47 (dd, 1H, $J$ = 3 and 12.8 Hz, $H_2$ chroman), 3.09 (dd, 1H, $J$ = 12.8 and 16.8 Hz, $H_3$ chroman), 2.90 (dd, 1H, $J$ = 3 and 16.8 Hz, $H_3$ chroman). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$: 190.24, 161.50, 142.20, 140.24, 134.91, 130.21, 129.05, 128.33, 126.30, 124.10, 122.71, 119.44, 118.29, 79.14, 44.32. Anal. Calcd for $C_{15}H_{10}Cl_2O_2$: C, 61.46; H, 3.44. Found: C, 61.39; H, 3.34.

*6.2.5. 7-Chloro-2-(4-chlorophenyl)chroman-4-one (**2e**)*

Yield 54%; m.p. 85–86 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 1677 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 7.86 (d, 2H, $J$ = 8.4 Hz, $H_{3'}$ and $H_{5'}$), 7.30–7.50 (m, 2H, $H_5$ and $H_6$ chroman), 7.08 (s, 1H, $H_8$ chroman), 7.05 (d, 2H, $J$ = 8.4 Hz, $H_{2'}$ and $H_{6'}$), 5.47 (dd, 1H, $J$ = 3 and 12.9 Hz, $H_2$ chroman), 3.03 (dd, 1H, $J$ = 12.9 and 16.8 Hz, $H_3$ chroman), 2.89 (dd, 1H, $J$ = 3 and 16.8 Hz, $H_3$ chroman). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$: 190.32, 161.51, 142.09, 136.70, 134.73, 129.07, 128.26, 127.44, 122.58, 119.40, 118.23, 79.16, 44.20. Anal. Calcd for $C_{15}H_{10}Cl_2O_2$: C, 61.46; H, 3.44. Found: C, 61.35; H, 3.41.

*6.2.6. 6-Chloro-2-(2-chlorophenyl)chroman-4-one (**2f**)*

Yield 57%; m.p. 110–112 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 1696 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 7.92 (s, 1H, $H_5$ chroman), 7.73 (d, 1H, $J$ = 8 Hz, $H_7$ chroman), 7.32–7.48 (m, 4H phenyl), 7.04 (d, 1H, $J$ = 8 Hz, $H_8$ chroman), 5.87 (dd, 1H, $J$ = 2.8 and 13.6 Hz, $H_2$ chroman), 3.05 (dd, 1H, $J$ = 2.8 and 17.6 Hz, $H_3$ chroman), 2.89 (dd, 1H, $J$ = 13.6 and 17.6 Hz, $H_3$ chroman). Anal. Calcd for $C_{15}H_{10}Cl_2O_2$: C, 61.46; H, 3.44. Found: C, 61.07; H, 3.46.

*6.2.7. 6-Chloro-2-(3-chlorophenyl)chroman-4-one (**2g**)*

Yield 58%; m.p. 129–130 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 1689 (C=O); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 7.86 (d, 1H, $J$ = 2.8 Hz, $H_5$ chroman), 7.49 (s, 1H, $H_{2'}$), 7.37 (m, 1H, $H_{5'}$), 7.30–7.36 (m, 2H, $H_{4'}$ and $H_{6'}$), 7.10

(d, 1H, $J = 7.2$ Hz, $H_8$ chroman), 7.05 (dd, 1H, $J = 2.8$ and 7.2 Hz, $H_7$ chroman), 5.47 (dd, 1H, $J = 3$ and 12.8 Hz, $H_2$ chroman), 3.09 (dd, 1H, $J = 12.8$ and 16.8 Hz, $H_3$ chroman), 2.90 (dd, 1H, $J = 3$ and 16.8 Hz, $H_3$ chroman). Anal. Calcd for $C_{15}H_{10}Cl_2O_2$: C, 61.46; H, 3.44. Found: C, 61.32; H, 3.32.

#### 6.2.8. 6-Chloro-2-(4-chlorophenyl)chroman-4-one (**2h**)

Yield 61%; m.p. 116–117 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 1693 (C=O); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$: 7.89 (d, 1H, $J = 2.8$ Hz, $H_5$ chroman), 7.47 (dd, 1H, $J = 2.8$ and 8.8 Hz, $H_7$ chroman), 7.37 (d, 2H, $J = 8.6$ Hz, $H_{3'}$ and $H_{5'}$), 7.10 (d, 1H, $J = 2.2$ Hz, $H_8$ chroman), 7.05 (d, 2H, $J = 8.6$ Hz, $H_{2'}$ and $H_{6'}$), 5.47 (dd, 1H, $J = 3$ and 12.8 Hz, $H_2$ chroman), 3.09 (dd, 1H, $J = 12.8$ and 16.8 Hz, $H_3$ chroman), 2.90 (dd, 1H, $J = 3$ and 16.8 Hz, $H_3$ chroman). $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$: 190.41, 159.94, 136.26, 131.64, 129.78, 127.99, 127.47, 127.13, 126.50, 121.72, 119.81, 76.99, 43.14. Anal. Calcd for $C_{15}H_{10}Cl_2O_2$: C, 61.46; H, 3.44. Found: C, 61.11; H, 3.15.

#### 6.2.9. 2-(2-Chlorophenyl)chroman-4-one (**2i**)

Yield 68%; m.p. 81–83 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 1672 (C=O); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$: 7.97 (d, 1H, $J = 8.4$ Hz, $H_5$ chroman), 7.76 (d, 1H, $J = 7.6$ Hz, $H_{3'}$), 7.53 (t, 1H, $J = 7.2$ Hz, $H_7$ chroman), 7.32–7.41 (m, 3H phenyl), 7.07–7.11 (m, 2H, $H_{6,8}$ chroman), 5.88 (dd, 1H, $J = 2.8$ and 13.2 Hz, $H_2$ chroman), 3.04 (dd, 1H, $J = 2.8$ and 16.8 Hz, $H_3$ chroman), 2.89 (dd, 1H, $J = 13.2$ and 16.8 Hz, $H_3$ chroman). Anal. Calcd for $C_{15}H_{11}ClO_2$: C, 69.64; H, 4.29. Found: C, 69.46; H, 4.13.

#### 6.2.10. 2-(3-Chlorophenyl)chroman-4-one (**2j**)

Yield 61%; m.p. 94–95 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 1664 (C=O); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$: 7.93 (d, 1H, $J = 8$ Hz, $H_5$ chroman), 7.55 (s, 1H, $H_{2'}$), 7.53 (t, 1H, $J = 7$ Hz, $H_7$ chroman), 7.34–7.38 (m, 3H phenyl), 7.06–7.10 (m, 2H, $H_{6,8}$ chroman), 5.46 (dd, 1H, $J = 2.8$ and 12.4 Hz, $H_2$ chroman), 3.03 (dd, 1H, $J = 12.4$ and 16.4 Hz, $H_3$ chroman), 2.89 (dd, 1H, $J = 2.8$ and 16.4 Hz, $H_3$ chroman). $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$: 191.36, 161.23, 140.78, 136.33, 134.83, 130.14, 128.87, 127.10, 126.34, 124.14, 121.87, 120.90, 118.10, 78.76, 44.65. Anal. Calcd for $C_{15}H_{11}ClO_2$: C, 69.64; H, 4.29. Found: C, 69.28; H, 4.38.

#### 6.2.11. 2-(4-Chlorophenyl)chroman-4-one (**2k**)

Yield 62%; m.p. 138–139 °C; IR (KBr, $cm^{-1}$) $\nu_{max}$: 1678 (C=O); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$: 7.93 (d, 1H, $J = 8$ Hz, $H_5$ chroman), 7.52 (t, 1H, $J = 8$ Hz, $H_7$ chroman), 7.39–7.44 (m, 4H phenyl), 7.04–7.09 (m, 2H, $H_{6,8}$ chroman), 5.47 (dd, 1H, $J = 2.8$ and 12.8 Hz, $H_2$ chroman), 3.04 (dd, 1H, $J = 12.8$ and 16.8 Hz, $H_3$ chroman), 2.88 (dd, 1H, $J = 2.8$ and 16.8 Hz, $H_3$ chroman). Anal. Calcd for $C_{15}H_{11}ClO_2$: C, 69.64; H, 4.29. Found: C, 69.63; H, 4.37.

### 6.3. Biology

#### 6.3.1. Cell lines and cell culture

Eight human cancer cell lines include MCF-7, MDA-MB-231 (human breast cancer), LNCaP, PC3 (human prostate cancer), Hep-G2 (human liver carcinoma), KB (human nasopharyngeal epidermoid carcinoma), SK-N-MC (human neuroblastoma) and K-562 (human erythroleukemic) were purchased from National Cell Bank of Iran (NCBI). The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (from GibcoeBRL, UK) and 100 mg/ml streptomycin and 100 U/ml penicillin at 37 °C in a humidified atmosphere with 5% $CO_2$ in air.

#### 6.3.2. In vitro cytotoxicity assay

The in vitro cytotoxic activity of the test compounds **2a–k** was assessed in comparison with etoposide using MTT colorimetric assay [26]. Briefly, cultures in the exponential growth phase were trypsinized and diluted in complete growth medium to give a total cell count of $5 \times 10^4$ cells/ml. The cell suspension (195 µl) was seeded into the 96-well plates (Nunc, Denmark). The plates were incubated overnight in a humidified air atmosphere at 37 °C with 5% $CO_2$. After plating, 5 µl of a serial dilution of each compound was added per well in triplicate. In each plate, there were three control wells (cells without test compounds) and three blank wells (the medium with 1% DMSO) for cell viability. Synthetic compounds were applied in DMSO, and the solvent reached a concentration not higher than 1% in all experiments. Etoposide was used as positive control for cytotoxicity. The plates were incubated for further 48 h. After treatment, the medium was removed and 200 µl phenol red-free medium containing MTT (1 mg/ml), was added to wells, followed by 4 h incubation. After incubation, the culture medium was replaced with 100 µl of DMSO and the absorbance of each well was measured by using a microplate reader (Gen5, Power wave xs2, BioTek, America) at 492 nm wavelengths. For each compound, the concentration causing 50% cell growth inhibition ($IC_{50}$) compared with the control was calculated from concentration–response curves by regression analysis.

#### 6.3.3. Acridine orange/ethidium bromide staining method

Apoptosis was determined morphologically after staining cells with acridine orange/ethidium bromide using fluorescence microscopy [27]. PC3 and MDA-MB-231 cells grown in 12-well plates (50,000 cells/well) were treated with and without $IC_{50}$ concentration of compounds **2d** and **2g** for 12 h. After washing three times with phosphate buffered saline (PBS), 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) were placed on a clean microscope slide and covered with a coverslip. The cells were immediately analyzed by fluorescence microscope (Axoscope 2 plus, Zeiss, Germany). All experiments were repeated three times and the cells with condensed or fragmented nuclei were counted as apoptotic cells.

#### 6.3.4. TUNEL assay

Apoptosis-induced nuclear DNA fragmentation was detected using *in situ* cell death detection kit (fluorescein, Roche) following the manufacturer's protocol. Briefly, 12 h following treating the PC3 and MDA-MB-231 cells with and without potent compounds **2d** and **2g** at concentrations of $IC_{50}$, cells were washed with PBS (pH 7.4) and fixed in 4% paraformaldehyde prepared freshly in PBS. The cells were again washed with PBS and permeabilised using 0.1% triton X 100 in 0.1% sodium citrate for 2 min on ice. After washing, the cells were incubated in the TdT incubation buffer (fluorescein/dNTP mix, TdT and labeling buffer) for 60 min at 37 °C in a humidified atmosphere in the dark and then washed and resuspended in 500 µl PBS. In humid chamber terminal deoxynucleotidyl transferase (TdT) catalyzes polymerization of fluorescein-labeled deoxynucleotides to free 3′-OH DNA ends in a template-independent manner. Free 3′-OH DNA in apoptotic cells was detected and quantified based on green fluorescence by flow cytometer (FACScan, LYSIS II, Becton Dickinson).

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