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Original article

Synthesis, in vitro cytotoxicity and apoptosis inducing study of 2-aryl-3-nitro-2*H*-chromene derivatives as potent anti-breast cancer agents

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ABSTRACT

A series of 2-aryl-3-nitro-2*H*-chromenes **4a**–**u** were designed as hybrid analogs of flavanone, β-nitrostyrene and nitrovinylstilbene scaffolds. They were synthesized from the reaction of appropriate β-nitrostyrenes and salicylaldehydes in good yields. In vitro cytotoxic activities of compounds **4a**–**u** were tested against breast cancer cell lines including MCF-7, T-47D and MDA-MB-231. Most compounds exhibited good cytotoxic activity against selected cell lines, being more potent than standard drug etoposide. Representatively, 8-methoxy-3-nitro-2-(4-chlorophenyl)-2*H*-chromene (**4l**) with $IC_{50} = 0.2$ μM against MCF-7 cells, was 36-times more potent than etoposide. Apoptosis as a mechanism of cell death for selected compounds **4h** and **4l** was confirmed morphologically by acridine orange/ethidium bromide double staining and TUNEL analysis, as well as caspase-3 activation assay.

1. Introduction

Cancer is one of the critical public health problems in the world. The corresponding incidence and mortality statistics shows that it is growing in developing as well as developed countries [1,2]. Despite significant advances in the diagnostic and therapeutic techniques, cancer is now the second most frequent cause of death after cardiovascular diseases [1,3].

Considering numerous reports and publications on the synthesis of anti-cancer agents, there is no drug with 100% efficacy. Therefore, there is still considerable demand for drug discovery leading to efficient anti-cancer compounds with new scaffold or specific mechanism of action to overcome the problems associated with current chemotherapeutics in cancer treatment, such as toxicity and drug resistance [4]. Among different strategies used for chemotherapy, induction of apoptosis or programmed cell death in cancer cells is one of the credible approaches to drug discovery and development of new anti-cancer agents [5,6].

Apoptosis plays an important role in the pathogenesis of several diseases including cancer (cancer initiation, progression and regulation of metastasis) [7,8]. It is well documented that resistance to apoptosis is one of the most important hallmarks of human cancers [9,10]. Three main types of cell death have been distinguished [11]; caspase-dependent apoptosis is the major form of controlled cell death in normal and cancer cells. It involves the activation of a family of cysteine proteases known as caspase. Caspase-3 is the key enzyme in promoting apoptotic pathway [12,13].

Several studies demonstrated that the mechanism of numerous anticancer agents occurs through the activation of apoptotic pathways in cancer cells [14–18]. Steifelder et al. have reported that the nitrovinyl side chain attached to an aromatic ring is an effective pharmacophore for the development of new pro-apoptotic agents (structure **A**, Fig. 1) [19]. On the other hand, structure–activity relationship study of resveratrol analogues showed that the methoxy groups at positions 3, 4 and 5 of the stilbene backbone

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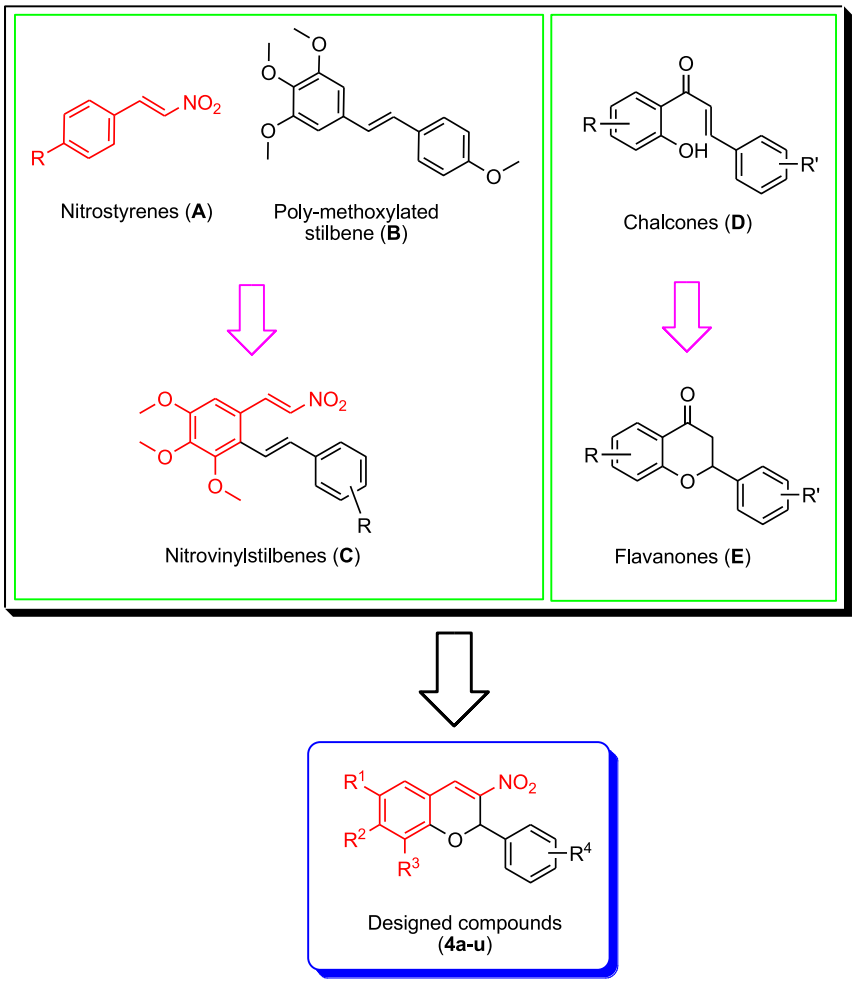


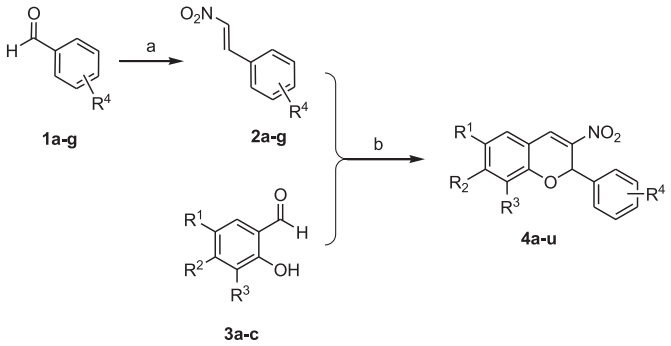
Fig. 1. Structural derivatization of some reported compounds (structures **A**–**E**) as pro-apoptotic anti-cancer agents. Compounds **4a**–**u** namely 2-aryl-3-nitro-2*H*-chromenes were designed in this work, as new pro-apoptotic anti-cancer agents.

(structure **B**, Fig. 1), greatly enhanced the pro-apoptotic effect and anti-cancer activity [20]. Also, Sreedhar et al. designed nitrovinylstilbenes (structure **C**, Fig. 1) as hybrid analogs of structures **A** and **B** exhibiting significant anti-proliferative activity with potential apoptosis induction ability in human cancer cells [21].

As a program aiming to develop flavanone and chromene derivatives as apoptosis inducers [22,23], we recently described a series of flavanones (structure **E**) derived from 2-hydroxychalcones (structure **D**, Fig. 1) as potential apoptosis-inducing agents [22]. The importance of chromene scaffold for antitumor activity [24], and the promising anti-proliferative and apoptosis induction activities of nitrovinylstilbenes (**C**) and flavanones (**E**) prompted us to hybridize their structural requirements. Thus we report here, synthesis, cytotoxicity and apoptosis-inducing activity of 2-aryl-3-nitro-2*H*-chromenes (**4a**–**u**, Fig. 1). Indeed, the designed compounds **4a**–**u** are cyclic rigid analogs of nitrovinylstilbenes (**C**) and originated from flavanones (**E**) by retaining the flavan scaffold in their core structure.

2. Chemistry

The synthetic pathway to obtain compounds namely 2-aryl-3-nitro-2*H*-chromene derivatives **4a**–**u** was outlined in Scheme 1. Initially, β-nitrostyrenes **2a**–**g** were prepared by



Scheme 1. Synthesis of compounds **4a**–**u**. *Reagents and conditions*: (a) nitromethane, *n*-butylamine, glacial acetic acid, reflux, 4 h; (b) DABCO, 40 °C, 2 h.

Henry–Knoevenagel condensation of different benzaldehydes **1a**–**g** with nitromethane, in the presence of *n*-butylamine. The desired β-nitrostyrenes **2a**–**g** were obtained in high yields and with little or no by-product [25]. The reaction of β-nitrostyrenes **2a**–**g** with appropriate salicylaldehyde **3a**–**c** in the presence of an organocatalyst base namely 1,4-diazabicyclo[2.2.2]octane (DABCO) afforded corresponding final compounds **4a**–**u** [26]. The required 2,3,4-trimethoxysalicylaldehyde (**3c**) was prepared in two steps

from commercially available 2,3,4-trimethoxybenzaldehyde (Scheme 2). The reaction of 2,3,4-trimethoxybenzaldehyde with hydrogen peroxide in the presence of H_2SO_4 in methanol afforded 2,3,4-trimethoxyphenol. The latter compound was converted to desired salicylaldehyde by using hexamethylenetetramine in refluxing TFA [27]. The structures of synthesized compounds were characterized by ^1H NMR, ^{13}C NMR, MS and CHN analysis as described in the experimental section.

3. Results and discussion

3.1. Biological study

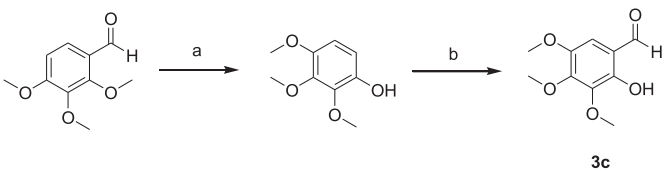
3.1.1. MTT assay

The in vitro cytotoxicity of synthesized compounds **4a**–**u** was evaluated against three different breast cancer cell lines MCF-7, T-47D and MDA-MB-231. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to determine the reduction in cancer cell viability induced by cytotoxic agents. The yellow tetrazolium is reduced in mitochondria by the action of dehydrogenase enzymes to an insoluble purple formazan product which quantified using spectrophotometric means [28,29]. The IC_{50} values of compounds **4a**–**u** in comparison to etoposide as standard drug are presented in Table 1. Results of the MTT assay revealed that all compounds showed potent activity (IC_{50}s $\leq$ 4.7 μM) against MCF-7 cells; their activities were superior to that of standard drug etoposide. The most potent compound **4l** possessing the IC_{50} value of 0.2 μM was 36-fold more active than etoposide against MCF-7. All compounds with the exception of **4i**, **4q**, **4t** and **4u**, exhibited higher cytotoxic activity against T-47D cell line respect to the standard drug. Although compound **4i** was as potent as etoposide against MDA-MB-231, but remaining compounds had superior activity in comparison to etoposide. The most potent compound (**4b**) against MDA-MB-231 (IC_{50} = 0.4 μM), was 32-times more active than etoposide.

Structurally, the designed compounds are divided to three series: un-substituted chromenes **4a**–**g**, 8-methoxychromones **4h**–**n** and 6,7,8-trimethoxychromenes **4o**–**u**. In each series, the substituent on the 2-phenyl ring was altered to optimize the cytotoxic activity against three different breast cancer cells. In the chromenes **4a**–**g**, all compounds showed same activities against MCF-7 cells and 3,4,5-trimethoxyphenyl derivative was better compound against T-47D cell line. Furthermore, 3,4-dimethoxyphenyl substituent showed more favorable cytotoxic activity against MDA-MB-231. The remaining substituents had same effect on this series, as shown by obtained IC_{50}s against MDA-MB-231.

Modification of substituents on the 2-phenyl ring in 8-methoxychromone **4h**–**n** series revealed that the 4-chloro group is preferred substituent for growth inhibitory activity against both MCF-7 and T-47D cell lines. Moreover, in 8-methoxychromone derivatives, the 4-nitro followed by 4-chloro groups improved the cytotoxicity against MDA-MB-231. The IC_{50} values of 6,7,8-trimethoxychromenes **4o**–**u** demonstrated that 3,4-dimethoxyphenyl moiety in compound **4p** offered the highest activity against T-47D cells while *para*-tolyl moiety was the best candidate for anti-proliferative activity towards MDA-MB-231 cell line.

The results suggested that the effect of methoxy substitution(s) on the chromene core directly depends on substituents on 2-phenyl ring. Consequently, the simultaneous presence of methoxy group at the 8-position of chromene ring and *para*-chloro on the 2-phenyl ring favored the antitumor activity.



Scheme 2. Synthesis of intermediate **3c**. *Reagents and conditions*: (a) H_2O_2, H_2SO_4, MeOH, 0 °C, 1 h; (b) HMTA, TFA, reflux, 2 h.

3.1.2. Acridine orange/ethidium bromide double staining

Since the main goal of our design was to obtain agents that promote or induce apoptosis, following the determination of the cytotoxicity by MTT assay, compound **4h** and **4l** with low IC_{50}s against MCF-7 cells were selected to determine apoptotic or necrotic cell death in MCF-7 and MDA-MB-231 cells. Apoptotic, necrotic and live cells can be distinguished using acridine orange/ethidium bromide (AO/EB) staining. Fluorescent DNA binding dyes AO and EB were used to detect the apoptotic and necrotic cells [30]. AO can penetrate living as well as dead cells and emits green fluorescence as a result of intercalation into double-stranded DNA while EB can only penetrate dead cells and emits red fluorescence after intercalation into DNA. The AO/EB staining which is sensitive to DNA was used to access changes in nuclear morphology. Analysis of the AO/EB staining of the selected compounds **4h** and **4l** in MCF-7 and MDA-MB-231 cell lines using fluorescence microscopy is shown in Fig. 2. In this figure, living cells has a normal green nucleus, but apoptotic cells show orange-stained nuclei with chromatin condensation or fragmentation, while necrotic cells appear uniformly orange-stained cell nuclei with no condensed chromatin. Analysis of the AO/EB staining revealed that the tested compounds **4h** and **4l** clearly exhibit late-stage apoptotic events (chromatin condensation and nuclear fragmentation) and result in reduced cell viability in human breast cancer cell lines.

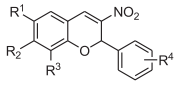
3.1.3. TUNEL assay

The TUNEL (TdT-mediated dUTP Nick-End Labeling; Promega) assay, has been designed to detect apoptotic cells by labeling 3′-OH ends of single- and double-stranded DNA fragments with fluorescein. This method was used for the detection of early stages of apoptosis (after 12 h treatment) in MDA-MB-231 cells to compare the ability of the compounds **4h** and **4l** to induce apoptosis and was quantified by flow-cytometry. As illustrated in Fig. 3, the apoptotic index of the test compounds was compared with negative control in MDA-MB-231 cells. The highest percentage of early apoptotic cells detected in this assay was 28%, resulting from treatment with the compound **4l** after 12 h of incubation. Exposure of MDA-MB-231 cells to IC_{50} concentration of compound **4h** induced apoptosis in 16% of the cells after 12 h. Besides, the corresponding value obtained after treatment with etoposide was 12% following 12 h of incubation. These data indicate that the cytotoxic activity of **4h** and **4l** in MDA-MB-231 cells occurs through apoptosis.

3.1.4. Caspase-3 activation assay

Caspase-3, one of the main executioner caspases, is activated in the apoptotic cells in both extrinsic and intrinsic pathways. The activation of caspase-3 is useful in the discovery of many potential anticancer agents. In an attempt to find out whether the induction of apoptosis by compound **4h** and **4l** is dependent on caspase-3 activity or not, we measured the activation of caspase-3 in MDA-MB-231 cells. Caspase-3 colorimetric assay was used to determine the increase in caspase-3 activity in early stage of apoptotic cells. The cleavage of the caspase 3-specific substrate that is labeled with the chromophore *p*-nitroaniline (*p*NA) by the activated caspase-3 leads to the releases of the chromophore *p*NA. Generation of free molecule *p*NA from apoptotic cells was quantitated

Table 1
Structures and in vitro cytotoxic activities of synthesized choromenes **4a**–**u** against breast cancer cell lines after 48 h treatment.



Compounds	R^1	R^2	R^3	R^4	Cytotoxicity (IC_{50} μM)[a]		
					MCF-7	T-47D	MDA-MB-231
4a	H	H	H	4-OCH_3	3.3 ± 0.9	3.9 ± 2.4	4.1 ± 0.1
4b	H	H	H	3,4-$(OCH_3)_2$	3.7 ± 0.2	3.2 ± 2.3	0.4 ± 0.2
4c	H	H	H	3,4,5-$(OCH_3)_3$	4.3 ± 0.6	2.1 ± 0.9	4.9 ± 1.0
4d	H	H	H	4-CH_3	3.7 ± 0.5	3.3 ± 2.5	4.6 ± 0.5
4e	H	H	H	4-Cl	3.7 ± 0.2	4.5 ± 1.2	4.1 ± 0.1
4f	H	H	H	4-NO_2	3.4 ± 0.3	4.5 ± 1.6	4.2 ± 0.1
4g	H	H	H	3,4-methylenedioxy	3.8 ± 0.4	5.6 ± 0.4	4.5 ± 0.4
4h	H	H	OCH_3	4-OCH_3	1.6 ± 0.2	3.9 ± 1.2	4.7 ± 0.6
4i	H	H	OCH_3	3,4-$(OCH_3)_2$	3.1 ± 0.6	9.1 ± 1.06	12.8 ± 2.3
4j	H	H	OCH_3	3,4,5-$(OCH_3)_3$	4.6 ± 0.5	6.2 ± 0.5	4.9 ± 0.1
4k	H	H	OCH_3	4-CH_3	3.9 ± 0.1	4.6 ± 0.3	3.3 ± 0.8
4l	H	H	OCH_3	4-Cl	0.2 ± 0	3.6 ± 2.1	2.0 ± 0.2
4m	H	H	OCH_3	4-NO_2	2.3 ± 0.5	7.5 ± 0.3	0.5 ± 0.2
4n	H	H	OCH_3	3,4-methylenedioxy	3.5 ± 0.07	6.3 ± 0.4	4.7 ± 0.4
4o	OCH_3	OCH_3	OCH_3	4-OCH_3	3.8 ± 0.2	4.7 ± 0.7	4.8 ± 0.6
4p	OCH_3	OCH_3	OCH_3	3,4-$(OCH_3)_2$	4.2 ± 0.1	3.0 ± 0.2	4.6 ± 0.1
4q	OCH_3	OCH_3	OCH_3	3,4,5-$(OCH_3)_3$	3.9 ± 0.3	12.7 ± 1.2	6.8 ± 0.1
4r	OCH_3	OCH_3	OCH_3	4-CH_3	3.1 ± 0.7	5.4 ± 1.3	3.6 ± 0.3
4s	OCH_3	OCH_3	OCH_3	4-Cl	3.5 ± 0.1	4.5 ± 0.4	5.2 ± 0.1
4t	OCH_3	OCH_3	OCH_3	4-NO_2	3.0 ± 1.4	10.3 ± 0.2	6.2 ± 0.7
4u	OCH_3	OCH_3	OCH_3	3,4-methylenedioxy	4.7 ± 0.8	9.5 ± 1.3	6.1 ± 0.4
Etoposide					7.2 ± 0.8	7.7 ± 0.7	12.8 ± 1.0

[a] Values are the mean ± SD. All experiments were performed at least three times.

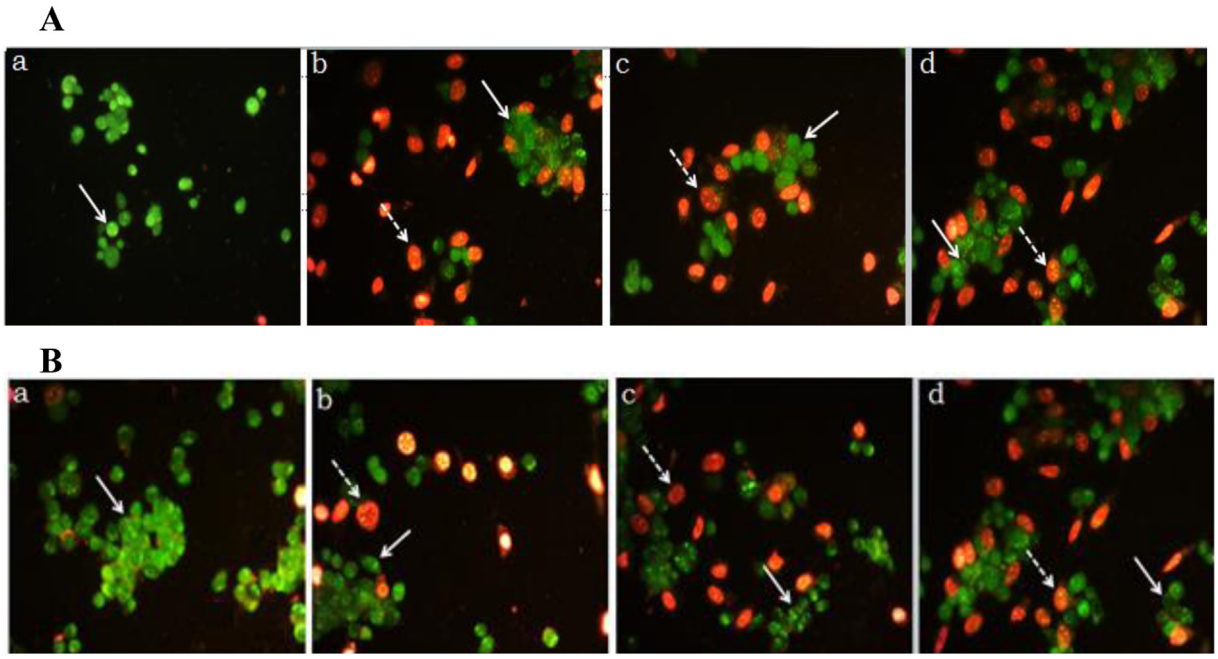


Fig. 2. Acridine orange/ethidium bromide double staining of MCF-7 (A) and MDA-MB-231 (B) cells with characteristic symptoms of apoptosis: a) DMSO 1% as control, b) cells after exposure to etoposide for 48 h, c) cells treated with compound **4l** for 48 h, d) cells treated with compound **4h** for 48 h. White arrow indicates live cells, dashed arrow shows apoptosis. The images of cells were taken with a fluorescence microscope at 400×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spectrophotometrically at 405 nm. A significant 3–4-fold increases was observed in the induction of caspase-3 in MDA-MB-231 cells treated with compounds **4l** and **4h** relative to the control (Fig. 4). These results suggested that the cytotoxic activity of **4h** and **4l** in MDA-MB-231 cells occurs via caspase-3-dependent apoptosis.

4. Conclusion

In conclusion, we designed 2-aryl-3-nitro-2*H*-chromenes **4a**–**u** as hybrid analogs of flavanones and β-nitrostyrenes which previously have been reported as apoptosis-inducing cytotoxic agents.

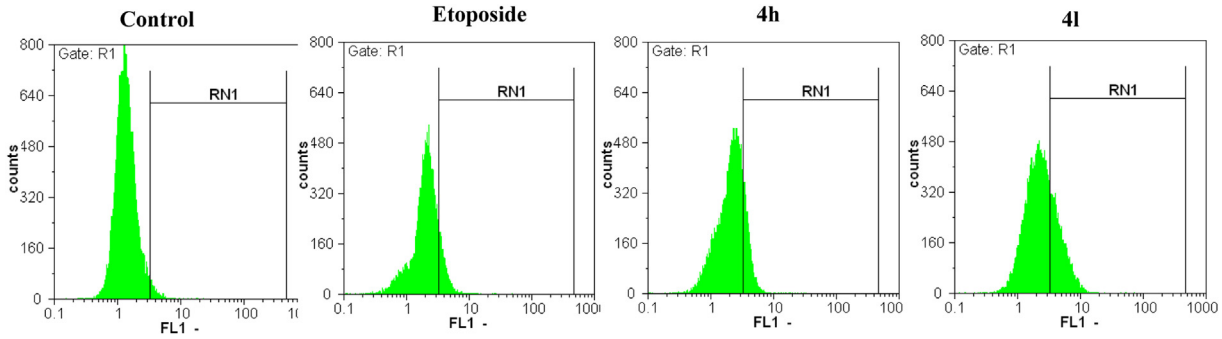


Fig. 3. Flow-cytometric analysis of MDA-MB-231 cell line. DNA fragmentation and generation of free 3′-OH DNA fragments was determined by TUNEL assay. MDA-MB-231 cells treated with DMSO 1% (negative control) or with IC_{50} values of etoposide (positive control) and compounds **4h** and **4l**, for 12 h. The presence of apoptotic cells is demonstrated by histogram analysis indicating increase in fluorescence intensity (RN1gate) in treated cells with synthetic compounds.

The compounds **4a**–**u** were easily synthesized from the reaction of appropriate β-nitrostyrenes and salicylaldehydes in good yields. Most of the synthesized compounds showed excellent cytotoxic activity against three breast cancer cell lines, more potent than standard drug etoposide. Representatively, compound **4l** with IC_{50} value of 0.2 μM was 36 times more potent than etoposide against MCF-7 cells. The SAR study demonstrated that *para*-chloro substituent on the 2-phenyl ring led to optimal effect in 8-methoxychromene derivatives. The most potent compounds against tested breast-cancer cell lines were belonging to the unsubstituted and 8-methoxylated chromene series. Meanwhile, the introduction of poly-methoxy substituents on 6,7,8 positions of chromene ring could not improve anti-proliferative activity against tested cell lines. Further biological assessments including flow-cytometric analysis, AO/EB staining, TUNEL and caspase-3 activation assays, revealed that the selected compounds **4h** and **4l** led to the induction of apoptosis through the activation of caspase-3 in breast cancer cell lines.

5. Experimental

5.1. Chemistry

Melting points are uncorrected and were determined with a Kofler hot-stage apparatus (Reichert, Vienna, Austria). ^1H and ^{13}C NMR spectra were recorded using Bruker 400 or 500 spectrometers. Chemical shifts (δ) are reported in ppm relative to TMS as internal standard. Coupling constant (J) values are presented in Hz and spin multiplicities are given as s (singlet), d (doublet), t (triplet) and m (multiplet). The IR spectra were obtained on a Nicolet Magna FTIR 550 spectrophotometer (potassium bromide disks). Mass spectra were determined on an Agilent Technology (HP) mass spectrometer operating at an ionization potential of 70 eV. The elemental analysis for C, H, N was carried out with an Elementar Analysen system GmbH VarioEL. All reagents and solvents used in this study were commercially available (from Merck chemical) and were used without further purification. The intermediate compounds β-nitrostyrenes **2a**–**g** and 2,3,4-trimethoxy salicylaldehyde (**3c**) were prepared according to the literature methods [25,27].

5.1.1. General procedure for the preparation of 2-aryl-3-nitro-2H-chromenes (*4a*–*u*)

A mixture of appropriate β-nitrostyrene **2a**–**g** (5 mmol), salicylaldehyde **3a**–**c** (10 mmol), and DABCO (2.5 mmol) was stirred at 40 °C for 4 h. Then the mixture was directly purified by column chromatography on silica gel eluting with *n*-hexane–EtOAc (7:3). The obtained product was recrystallized from ethanol to give desired compound **4**.

*5.1.1.1. 3-Nitro-2-(4-methoxyphenyl)-2H-chromene (**4a**).* Yield 72%; mp 94–96 °C; IR (KBr, cm^{-1}): ν 1644, 1608, 1511, 1454, 1326, 1255, 1168, 1111, 1060, 753; ^1H NMR (500 MHz, $CDCl_3$): δ 3.77 (s, 3H, OCH_3), 6.53 (s, 1H, H_2-chromene), 6.83–6.86 (m, 3H, H_8-chromene and $H_{3,5}$-phenyl), 7.01 (dt, 1H, J = 7.5 and 1.0 Hz, H_6-chromene), 7.29–7.31 (m, 2H, $H_{2,6}$-phenyl), 7.32–7.34 (m, 2H, $H_{5,7}$-chromene), 8.05 (s, 1H, H_4-chromene). ^{13}C NMR (125 MHz, $CDCl_3$): δ 56.39, 73.08, 107.36, 117.31, 117.63, 123.29, 124.12, 127.84, 130.08, 130.81, 134.95, 140.27, 143.52, 148.37, 153.09. Anal. Calcd for $C_{16}H_{13}NO_4$: C, 67.84; H, 4.63; N, 4.94. Found: C, 67.95; H, 4.71; N, 4.75.

*5.1.1.2. 3-Nitro-2-(3,4-dimethoxyphenyl)-2H-chromene (**4b**).* Yield 90%; mp 87–89 °C; IR (KBr, cm^{-1}): ν 1646, 1601, 1514, 1449, 1413, 1331, 1253, 1142, 1024, 748; ^1H NMR (500 MHz, $CDCl_3$): δ 3.77 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 6.46 (s, 1H, H_2-chromene), 6.48 (d, 1H, J = 7.5 Hz, H_5-phenyl), 6.84–6.88 (m, 3H, H_8-chromene and $H_{2,6}$-phenyl), 7.02 (t, 1H, J = 7.5 Hz, H_6-chromene), 7.29–7.34 (m, 2H, $H_{5,7}$-chromene), 8.04 (s, 1H, H_4-chromene). ^{13}C NMR (125 MHz, $CDCl_3$): δ 55.23, 56.87, 73.11, 107.39, 117.19, 117.64, 123.09, 123.97, 128.07, 130.52, 130.78, 134.65, 140.12, 143.61, 148.40. MS (*m*/*z*, %): 313 (m^+, 36), 267 (100), 251 (18), 223 (23), 205 (7), 181 (8), 165 (10), 152 (13). Anal. Calcd for $C_{17}H_{15}NO_5$: C, 65.17; H, 4.83; N, 4.47. Found: C, 65.35; H, 4.76; N, 4.35.

*5.1.1.3. 3-Nitro-2-(3,4,5-trimethoxyphenyl)-2H-chromene (**4c**).* Yield 81%; mp 124–126 °C; IR (KBr, cm^{-1}): ν 1644, 1593, 1501, 1454, 1321, 1239, 1122, 1004, 830, 753; ^1H NMR (500 MHz, $CDCl_3$): δ 3.77 (s, 6H, 2 × OCH_3), 3.81 (s, 3H, OCH_3), 6.53 (s, 1H, H_2-chromene), 6.58 (s, 2H, $H_{2,6}$-phenyl), 6.91 (d, 1H, J = 8.0 Hz, H_8-chromene), 7.01–7.05 (m, 1H, H_6-chromene), 7.34–7.38 (m, 2H, $H_{5,7}$-chromone), 8.07 (s, 1H, H_4-chromene). ^{13}C NMR (125 MHz, $CDCl_3$): δ 56.19, 56.25, 56.42, 73.80, 104.18, 107.21, 113.28, 129.61, 131.83, 140.05, 142.11, 142.79, 147.88, 148.75, 153.38. MS (*m*/*z*, %): 343 (m^+, 52), 297 (100), 281 (8), 264 (18), 253 (14), 239 (11), 168 (22), 139 (16). Anal. Calcd for $C_{18}H_{17}NO_6$: C, 62.97; H, 4.99; N, 4.08. Found: C, 63.15; H, 4.71; N, 4.15.

*5.1.1.4. 3-Nitro-2-(4-methylphenyl)-2H-chromene (**4d**).* Yield 67%; mp 120–122 °C; IR (KBr, cm^{-1}): ν 1650, 1602, 1510, 1455, 1324, 1198, 1116, 1060, 819, 750; ^1H NMR (500 MHz, $CDCl_3$): δ 2.31 (s, 3H, CH_3), 6.55 (s, 1H, H_2-chromene), 6.86 (d, 1H, J = 7.7 Hz, H_8-chromene), 7.01 (dt, 1H, J = 7.7 and 1.0 Hz, H_6-chromene), 7.13 (d, 2H, J = 7.7 Hz, $H_{3,5}$-phenyl), 7.26 (d, 2H, J = 7.7 Hz, $H_{2,6}$-phenyl), 7.30–7.34 (m, 2H, $H_{5,7}$-chromone), 8.05 (s, 1H, H_4-chromene). ^{13}C NMR (125 MHz,

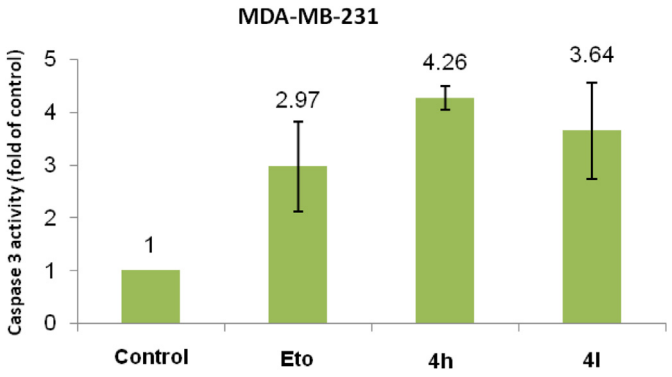


Fig. 4. Effect of etoposide (positive control), compounds **4h** and **4l** on caspase-3 activity in MDA-MB-231 cell line compared to the negative control (untreated cells) after 12 h. Caspase-3 activities were determined by mixing the cell lysates with DEVD-*p*NA and monitoring colorimetric substrate hydrolysis at 405 nm using absorbance plate reader. The values are shown as a fold-increase compared to the control.

$CDCl_3$): δ 21.18, 73.13, 107.41, 117.62, 118.55, 129.43, 129.87, 130.12, 134.76, 135.52, 139.68, 141.46, 142.67, 147.93, 148.74. MS (*m*/*z*, %): 267 (m^+, 39), 250 (11), 221 (100), 205 (15), 191 (10), 178 (34), 165 (9), 115 (7). Anal. Calcd for $C_{16}H_{13}NO_3$: C, 71.90; H, 4.90; N, 5.24. Found: C, 71.65; H, 4.71; N, 5.33.

*5.1.1.5. 3-Nitro-2-(4-chlorophenyl)-2H-chromene (**4e**).* Yield 80%; mp 118–120 °C; IR (KBr, cm^{-1}): ν 1641, 1598, 1487, 1448, 1325, 1221, 1147, 1063, 1006, 753; ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.56 (s, 1H, H_2-chromene), 6.87 (d, 1H, J = 7.5 Hz, H_8-chromene), 7.03 (dt, 1H, J = 7.5 and 1.05 Hz, H_6-chromene), 7.28–7.30 (m, 2H, $H_{3,5}$-phenyl), 7.31–7.33 (m, 2H, $H_{2,6}$-phenyl), 7.34–7.36 (m, 2H, $H_{5,7}$-chromene), 8.07 (s, 1H, H_4-chromene). ^{13}C NMR (125 MHz, $CDCl_3$): δ 73.09, 107.38, 117.53, 118.36, 128.43, 129.81, 130.03, 134.73, 135.46, 139.68, 141.56, 142.67, 147.93, 148.70. Anal. Calcd for $C_{15}H_{10}ClNO_3$: C, 62.62; H, 3.50; N, 4.87. Found: C, 62.84; H, 3.71; N, 4.69.

*5.1.1.6. 3-Nitro-2-(4-nitrophenyl)-2H-chromene (**4f**).* Yield 55%; mp 129–131 °C; IR (KBr, cm^{-1}): ν 1651, 1603, 1525, 1455, 1345, 1194, 1153, 1115, 1066, 753; ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.67 (s, 1H, H_2-chromene), 6.92 (d, 1H, J = 8.0 Hz, H_8-chromene), 7.06 (dt, 1H, J = 8.0 and 1.0 Hz, H_6-chromene), 7.36–7.40 (m, 2H, $H_{5,7}$-chromene), 7.58 (d, 2H, J = 8.0 Hz, $H_{2,6}$-phenyl), 8.11 (s, 1H, H_4-chromene), 8.19 (d, 2H, J = 8.0 Hz, $H_{3,5}$-phenyl). ^{13}C NMR (125 MHz, $CDCl_3$): δ 73.03, 107.31, 117.25, 117.52, 123.19, 124.09, 127.97, 130.02, 130.77, 134.84, 140.19, 143.53, 148.42, 153.05. Anal. Calcd for $C_{15}H_{10}N_2O_5$: C, 60.41; H, 3.38; N, 9.39. Found: C, 60.33; H, 3.71; N, 9.38.

*5.1.1.7. 3-Nitro-2-(3,4-methylenedioxyphenyl)-2H-chromene (**4g**).* Yield 85%; mp 97–99 °C; IR (KBr, cm^{-1}): ν 1646, 1603, 1506, 1447, 1323, 1257, 1222, 1107, 1035, 749; ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.94 (s, 2H, OCH_2O), 6.49 (s, 1H, H_2-chromene), 6.74 (d, 1H, J = 8.0 Hz, H_5-phenyl), 6.85–6.87 (m, 3H, H_8-chromene and $H_{2,6}$-phenyl), 7.02 (t, 1H, J = 8.0 Hz, 1H, H_6-chromene), 7.32–7.34 (m, 2H, $H_{5,7}$-chromone), 8.05 (s, 1H, H_4-chromene). ^{13}C NMR (125 MHz, $CDCl_3$): δ 73.95, 95.08, 102.13, 107.67, 108.53, 117.09, 121.49, 124.37, 126.64, 129.83, 135.32, 138.97, 148.45, 153.27. MS (*m*/*z*, %): 297 (m^+, 31), 251 (100), 221 (10), 193 (17), 165 (42), 146 (8), 139 (12). Anal. Calcd for $C_{16}H_{11}NO_5$: C, 64.65; H, 3.73; N, 4.71. Found: C, 64.41; H, 3.71; N, 4.93.

*5.1.1.8. 8-Methoxy-3-nitro-2-(4-methoxyphenyl)-2H-chromene (**4h**).* Yield 82%; mp 122–124 °C; IR (KBr, cm^{-1}): ν 1641, 1605, 1575, 1508, 1477, 1320, 1269, 1172, 1055, 755; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.82 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 6.65 (s, 1H, H_2-chromene), 6.94–6.98 (m, 3H, $H_{5,6,7}$-chromene), 7.12 (d, 2H, J = 8.0 Hz, $H_{3,5}$-phenyl), 7.30 (d, 2H, J = 8.0 Hz, $H_{2,6}$-phenyl), 8.05 (s, 1H, H_4-chromene). ^{13}C NMR (125 MHz, $CDCl_3$): δ 56.12, 56.31, 73.89, 104.01, 116.47, 118.75, 121.87, 122.59, 129.18, 132.08, 139.01, 141.59, 142.75, 148.58, 153.41. MS (*m*/*z*, %): 313 (M^+, 4), 297 (42), 267 (26), 251 (100), 236 (15), 208 (30), 165 (38). Anal. Calcd for $C_{17}H_{15}NO_5$: C, 65.17; H, 4.83; N, 4.47. Found: C, 65.39; H, 4.71; N, 4.36.

*5.1.1.9. 8-Methoxy-3-nitro-2-(3,4-dimethoxyphenyl)-2H-chromene (**4i**).* Yield 89%; mp127–129 °C; IR (KBr, cm^{-1}): ν 1649, 1599, 1575, 1509, 1474, 1325, 1260, 1140, 1025, 750; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.83 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 6.63 (s, 1H, H_2-chromene), 6.75 (d, 1H, J = 8.0 Hz, H_7-chromene), 6.87 (dd, 1H, J = 8.0 and 2.0 Hz, H_6-chromene), 6.95–6.99 (m, 3H, $H_{2,5,6}$-phenyl), 7.04 (d, 1H, J = 8.0 Hz, H_5-chromene), 8.06 (s, 1H, H_4-chromene), ^{13}C NMR (100 MHz, $CDCl_3$): δ 56.06, 56.26, 60.77, 74.05, 103.98, 116.59, 118.86, 121.98, 122.63, 129.22, 132.03, 138.78, 141.53, 142.70, 148.66, 153.34. Anal. Calcd for $C_{18}H_{17}NO_6$: C, 62.97; H, 4.99; N, 4.08. Found: C, 62.78; H, 4.81; N, 4.14.

*5.1.1.10. 8-Methoxy-3-nitro-2-(3,4,5-trimethoxyphenyl)-2H-chromene (**4j**).* Yield 70%; mp 131–133 °C; IR (KBr, cm^{-1}): ν 1651, 1602, 1509, 1450, 1339, 1268, 1126, 1048, 860, 786; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.78 (s, 6H, 2 × OCH_3), 3.81 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 6.63 (s, 1H, H_2-chromene), 6.64 (s, 2H, $H_{2,6}$-phenyl), 6.95–6.99 (m, 3H, $H_{5,6,7}$-chromene), 8.06 (s, 1H, H_4-chromene). ^{13}C NMR (100 MHz, $CDCl_3$): δ 56.08, 56.22, 60.73, 61.67, 74.03, 103.87, 115.99, 118.82, 122.08, 122.67, 129.31, 131.95, 138.75, 141.48, 142.70, 148.59, 153.29. Anal. Calcd for $C_{19}H_{19}NO_7$: C, 61.12; H, 5.13; N, 3.75. Found: C, 61.35; H, 5.38; N, 3.82.

*5.1.1.11. 8-Methoxy-3-nitro-2-(4-methylphenyl)-2H-chromene (**4k**).* Yield 74%; mp 125–127 °C; IR (KBr, cm^{-1}): ν 1645, 1608, 1574, 1512, 1481, 1323, 1268, 1212, 1055, 750; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.31 (s, 3H, CH_3), 3.81 (s, 3H, OCH_3), 6.64 (s, 1H, H_2-chromene), 6.93–6.97 (m, 3H, $H_{5,6,7}$-chromene), 7.12 (d, 2H, J = 7.7 Hz, $H_{3,5}$-phenyl), 7.30 (d, 2H, J = 7.7 Hz, $H_{2,6}$-phenyl), 8.04 (s, 1H, H_4-chromene). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.28, 60.68, 74.03, 103.85, 116.60, 118.92, 121.84, 122.63, 129.25, 132.07, 139.18, 141.53, 142.66, 148.73, 149.57. Anal. Calcd for $C_{17}H_{15}NO_4$: C, 68.68; H, 5.09; N, 4.71. Found: C, 68.95; H, 5.34; N, 4.63.

*5.1.1.12. 8-Methoxy-3-nitro-2-(4-chlorophenyl)-2H-chromene (**4l**).* Yield 92%; mp 130–132 °C; IR (KBr, cm^{-1}): ν 1651, 1598, 1577, 1512, 1478, 1317, 1265, 1187, 1082, 783; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.84 (s, 3H, OCH_3), 6.64 (s, 1H, H_2-chromene), 6.96–7.00 (m, 3H, $H_{5,6,7}$-chromene), 7.29–7.31 (m, 2H, $H_{3,5}$-phenyl), 7.34–7.36 (m, 2H, $H_{2,6}$-phenyl), 8.06 (s, 1H, H_4-chromene). ^{13}C NMR (100 MHz, $CDCl_3$): δ 56.42, 74.03, 104.05, 116.60, 118.74, 121.87, 122.63, 129.43, 132.19, 139.22, 141.73, 142.56, 148.67, 149.83. MS (*m*/*z*, %): 319 ($[M+2]^+$, 20), 317 (M^+, 48), 271 (100), 256 (12), 228 (22), 178 (18), 165 (50). Anal. Calcd for $C_{16}H_{12}ClNO_4$: C, 60.48; H, 3.81; N, 4.41. Found: C, 60.35; H, 3.77; N, 4.56.

*5.1.1.13. 8-Methoxy-3-nitro-2-(4-nitrophenyl)-2H-chromene (**4m**).* Yield 60%; mp 138–140 °C; IR (KBr, cm^{-1}): ν 1654, 1608, 1574, 1512, 1516, 1478, 1348, 1268, 1058, 857; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.84 (s, 3H, OCH_3), 6.67 (s, 1H, H_2-chromene), 6.96–6.99 (m, 3H, $H_{5,6,7}$-chromene), 7.30 (d, 2H, J = 8.4 Hz, $H_{2,6}$-phenyl), 8.02 (s, 1H, H_4-chromene), 8.19 (d, 2H, J = 8.4 Hz, $H_{3,5}$-phenyl). ^{13}C NMR (125 MHz, $CDCl_3$): δ 56.29, 73.09, 104.01, 116.95, 117.72, 123.32, 124.16, 128.07, 130.09, 131.23, 134.64, 140.53, 143.76, 148.62, 153.07. MS (*m*/*z*, %): 328 (m^+, 38), 282 (100), 236 (46), 193 (45), 165 (73), 152 (42), 139

(42), 63 (46). Anal. Calcd for $C_{16}H_{12}N_2O_6$: C, 58.54; H, 3.68; N, 8.53. Found: C, 58.35; H, 3.71; N, 8.32.

*5.1.1.14. 8-Methoxy-3-nitro-2-(3,4-methylenedioxyphenyl)-2H-chromene (**4n**).* Yield 65%; mp 143–136 °C; IR (KBr, cm^{-1}): ν 1649, 1602, 1574, 1496, 1448, 1330, 1266, 1101, 1034, 971; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.83 (s, 3H, OCH_3), 5.94 (s, 2H, OCH_2O), 6.64 (s, 1H, H_2-chromene), 6.76 (d, 1H, $J = 8$ Hz, H_7-chromene), 6.87 (dd, 1H, $J = 8.0$ and 2 Hz, H_6-chromene), 6.96–7.01 (m, 3H, $H_{2,5,6}$-phenyl), 7.04 (d, 1H, $J = 8.0$ Hz, H_5-chromene), 8.04 (s, 1H, H_4-chromene). ^{13}C NMR (100 MHz, $CDCl_3$): δ 56.42, 73.85, 95.05, 102.06, 107.53, 108.43, 121.34, 124.23, 126.64, 129.59, 135.40, 139.17, 148.13, 153.27. MS (*m*/*z*, %): 327 (m^+, 18), 281 (100), 266 (9), 251 (5), 238 (13), 208 (6), 152 (22). Anal. Calcd for $C_{17}H_{13}NO_6$: C, 62.39; H, 4.00; N, 4.28. Found: C, 62.45; H, 4.18; N, 4.51.

*5.1.1.15. 6,7,8-Trimethoxy-3-nitro-2-(4-methoxyphenyl)-2H-chromene (**4o**).* Yield 65%; mp 130–132 °C; IR (KBr, cm^{-1}): ν 1642, 1608, 1507, 1461, 1422, 1379, 1309, 1247, 1027, 750; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.63 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 6.57 (s, 1H, H_2-chromene), 6.61 (s, 1H, H_5-chromene), 6.83 (d, 2H, $J = 8.5$ Hz, $H_{3,5}$-phenyl), 7.32 (d, 2H, $J = 8.5$ Hz, $H_{2,6}$-phenyl), 8.02 (s, 1H, H_4-chromene). ^{13}C NMR (125 MHz, $CDCl_3$): δ 56.18, 56.29, 56.41, 60.85, 73.82, 104.18, 107.24, 113.28, 129.49, 131.65, 138.81, 140.12, 141.99, 142.53, 147.76, 148.59, 153.41. MS (*m*/*z*, %): 373 (m^+, 14), 327 (100), 311 (22), 297 (20), 283 (21), 266 (8), 155 (20), 139 (12), 127 (16). Anal. Calcd for $C_{19}H_{19}NO_7$: C, 61.12; H, 5.13; N, 3.75. Found: C, 61.35; H, 5.41; N, 3.58.

*5.1.1.16. 6,7,8-Trimethoxy-3-nitro-2-(3,4-dimethoxyphenyl)-2H-chromene (**4p**).* Yield 60%; mp 140–142 °C; IR (KBr, cm^{-1}): ν 1654, 1580, 1521, 1453, 1425, 1265, 1113, 1030, 848,756; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.65 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.85 (s, 6H, 2 × OCH_3), 3.94 (s, 3H, OCH_3), 6.57 (s, 1H, H_2-chromene), 6.61 (s, 1H, H_5-chromene), 6.75 (d, 1H, $J = 8.3$ Hz, H_5-phenyl), 6.86 (d, 1H, $J = 8.3$ Hz, H_6-phenyl), 7.01 (s, 1H, H_2-phenyl), 8.02 (s, 1H, H_4-chromene). ^{13}C NMR (125 MHz, $CDCl_3$): δ 55.83, 55.92, 56.41, 61.42, 61.52, 73.80, 107.19, 110.54, 110.80, 113.43, 119.25, 128.49, 129.50, 140.23, 141.84, 142.80, 147.70, 148.57, 149.14, 149.93. Anal. Calcd for $C_{20}H_{21}NO_8$: C, 59.55; H, 5.25; N, 3.47. Found: C, 59.35; H, 5.51; N, 3.35.

*5.1.1.17. 6,7,8-Trimethoxy-3-nitro-2-(3,4,5-trimethoxyphenyl)-2H-chromene (**4q**).* Yield 54%; mp 147–149 °C; IR (KBr, cm^{-1}): ν 1642, 1592, 1508, 1458, 1421, 1278, 1129, 1108, 1064, 968; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.72 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.85 (s, 6H, 2 × OCH_3), 3.95 (s, 3H, OCH_3), 6.58 (s, 1H, H_2-chromene), 6.61 (s, 3H, H_5-chromene, $H_{2,6}$-phenyl), 8.03 (s, 1H, H_4-chromene). ^{13}C NMR (125 MHz, $CDCl_3$): δ 56.14, 56.27, 56.42, 60.79, 61.42, 61.56, 73.80, 104.10, 107.21, 113.24, 129.57, 131.73, 138.87, 140.09, 142.01, 142.71, 147.82, 148.68, 153.36. MS (*m*/*z*, %): 433 (M^+, 23), 387 (100), 357 (26), 343 (9), 329 (7), 311 (10), 157 (12). Anal. Calcd for $C_{21}H_{23}NO_9$: C, 58.20; H, 5.35; N, 3.23. Found: C, 58.35; H, 5.41; N, 3.22.

*5.1.1.18. 6,7,8-Trimethoxy-3-nitro-2-(4-methylphenyl)-2H-chromene (**4r**).* Yield 90%; mp 134–136 °C; IR (KBr, cm^{-1}): ν 1642, 1608, 1564, 1483, 1423, 1378, 1308, 1271, 1115, 992; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.31 (s, 3H, CH_3), 3.65 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 6.59 (s, 1H, H_2-chromene), 6.61 (s, 1H, H_5-chromene), 7.12 (d, 2H, $J = 7.8$ Hz, $H_{3,5}$-phenyl), 7.28 (d, 2H, $J = 7.8$ Hz, $H_{2,6}$-phenyl), 8.02 (s, 1H, H_4-chromene). ^{13}C NMR (125 MHz, $CDCl_3$): δ 21.19, 56.39, 61.40, 61.44, 73.85, 107.26, 113.39, 114.10, 127.01, 128.56, 129.46, 129.54, 133.12, 139.48, 140.21, 141.89, 142.74, 147.66, 148.51. Anal. Calcd for $C_{19}H_{19}NO_6$: C, 63.86; H, 5.36; N, 3.92. Found: C, 63.65; H, 5.51; N, 3.73.

*5.1.1.19. 6,7,8-Trimethoxy-3-nitro-2-(4-chlorophenyl)-2H-chromene (**4s**).* Yield 87%; mp 139–141 °C; IR (KBr, cm^{-1}): ν 1638, 1602, 1564, 1486, 1458, 1423, 1378, 1270, 1116, 829; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.68 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.59 (s, 1H, H_2-chromene), 6.61 (s, 1H, H_5-chromene), 7.30 (d, 2H, $J = 8.1$ Hz, $H_{3,5}$-phenyl), 7.34 (d, 2H, $J = 8.1$ Hz, $H_{2,6}$-phenyl), 8.03 (s, 1H, H_4-chromene). ^{13}C NMR (125 MHz, $CDCl_3$): δ 56.39, 61.39, 61.47, 73.16, 107.30, 113.03, 128.38, 129.01, 129.80, 134.70, 135.41, 139.68, 141.63, 142.67, 147.88, 148.71. Anal. Calcd for $C_{18}H_{16}ClNO_6$: C, 57.23; H, 4.27; N, 3.71. Found: C, 57.39; H, 4.33; N, 3.77.

*5.1.1.20. 6,7,8-Trimethoxy-3-nitro-2-(4-nitrophenyl)-2H-chromene (**4t**).* Yield 50%; mp 147–149 °C; IR (KBr, cm^{-1}): ν 1645, 1576, 1481, 1451, 1339, 1254, 1228, 1145, 1039, 753; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.68 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 6.58 (s, 1H, H_2-chromene), 6.61 (s, 1H, H_5-chromene), 7.57 (d, 2H, $J = 8.5$ Hz, $H_{2,6}$-phenyl), 8.02 (s, 1H, H_4-chromene), 8.19 (d, 2H, $J = 8.5$ Hz, $H_{3,5}$-phenyl). ^{13}C NMR (125 MHz, $CDCl_3$): δ 56.42, 61.34, 61.55, 73.22, 107.35, 113.13, 128.44, 128.93, 129.79, 134.68, 135.40, 139.63, 141.56, 142.65, 148.03, 148.61, 153.08. MS (*m*/*z*, %): 388 (m^+, 4), 342 (10), 212 (100), 197 (89), 169 (80), 154 (27), 126 (41), 83 (21). Anal. Calcd for $C_{18}H_{16}N_2O_8$: C, 55.67; H, 4.15; N, 7.21. Found: C, 55.58; H, 4.41; N, 7.23.

*5.1.1.21. 6,7,8-Trimethoxy-3-nitro-2-(3,4-methylenedioxyphenyl)-2H-chromene (**4u**).* Yield 75%; mp 150–152 °C; IR (KBr, cm^{-1}): ν 1648, 1561, 1485, 1457, 1319, 1270, 1139, 1036, 1039, 987; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.66 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 5.88 (s, 2H, OCH_2O), 6.56 (s, 1H, H_2-chromene), 6.62 (s, 1H, H_5-chromene), 6.76 (d, 1H, $J = 8.3$ Hz, H_5-phenyl), 6.85 (d, 1H, $J = 8.3$ Hz, H_6-phenyl), 7.01 (s, 1H, H_2-phenyl), 8.05 (s, 1H, H_4-chromene). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.78, 55.91, 60.25, 73.83, 96.23, 102.35, 107.59, 108.63, 113.75, 121.44, 124.30, 126.64, 129.59, 135.40, 139.35, 148.17, 153.38. Anal. Calcd for $C_{19}H_{17}NO_8$: C, 58.92; H, 4.42; N, 3.62. Found: C, 58.78; H, 4.71; N, 3.35.

5.2. Biology

5.2.1. Cell lines and cell culture

Three different breast cancer cell lines including MCF-7, T-47D and MDA-MB-231 were purchased from National Cell Bank of Iran (NCBI). The cells were routinely maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (from GibcoeBRL, UK) and 100 μg/mL streptomycin and 100 U/mL penicillin at 37 °C in a humidified incubator with 5% CO_2.

5.2.2. In vitro cytotoxicity assay

The synthesized compounds **4a**–**u** were tested against three human breast cancer cell lines using MTT colorimetric assay [28,29]. Briefly, cells in the log-phase of growth were harvested by trypsinization, and diluted in complete growth medium to give a total cell count of 5×10^4 cells/mL. The cell suspension (195 μL) from different cell lines was seeded in 96-well plates (Nunc, Denmark) and the plates were incubated overnight in a humidified air atmosphere at 37 °C with 5% CO_2. After plating, 5 μL of the media containing various concentrations of the compounds 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) as negative controls for cell viability. Synthetic compounds were initially dissolved in DMSO, and the final concentration of DMSO was less than 1% in all experiments. Etoposide was used as positive control for cytotoxicity. After 48 h further

incubation, the medium was removed and 200 μL phenol red-free medium containing MTT (1 mg/mL, final concentration), was added to wells, followed by 4 h incubation. Then, the culture medium was replaced with 100 μL of DMSO and the absorbance values were determined at 492 nm using a multi-well plate reader (Gen5, Power wave xs2, BioTek, America). The IC_{50} values compared with the control were calculated by nonlinear regression analysis and expressed in mean ± SD.

5.2.3. AO/EB staining method

Apoptosis in treated cancer cells was determined morphologically after staining with acridine orange/ethidium bromide (AO/EB) using fluorescence microscopy [30]. MDA-MB-231 cell grown in 12-well plates (50,000 cells/well) were treated with and without IC_{50} concentrations of compounds **4h** and **4l** for 48 h. After washing three times with phosphate buffered saline (PBS), 9 μL of cell suspension were stained with 1 μL of dye mixture (100 mg/mL AO and 100 mg/mL EB in PBS). Stained cell suspension (10 μL) were placed on a clean microscope slide and covered with a coverslip. The suspension was immediately examined by fluorescence microscope (Axoscope 2 plus, Zeiss, Germany). All experiments were repeated three times.

5.2.4. TUNEL assay

Apoptosis-induced nuclear DNA fragmentation in early stage of apoptosis was detected using in situ cell death detection kit (fluorescein, Roche) following the manufacturer's protocol. Briefly, after 12 h treating the MDA-MB-231 cell with and without IC_{50} concentrations of compounds **4l** and **4h**, 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 permeabilized using 0.1% triton X 100 in sodium citrate 0.1% for 2 min on ice. Following 2 times washes in PBS buffer, 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 mL PBS. In humid chamber, terminal deoxynucleotidyltransferase (TdT) catalyzes polymerization of fluorescein-labeled deoxynucleotides to free 3′-OH DNA ends in the absence of a template. Free 3′-OH DNA in apoptotic cells was detected and quantified based on green fluorescence by flow cytometer (FACScan, LYSIS II, Becton Dickinson).

5.2.5. Caspase-3 activation assay

The activity of caspase-3 was determined in early stage of apoptosis (after 12 h) using caspase-3 colorimetric assay [31]. Briefly, MDA-MB-231 cells (5×10^5 cells/well) in 6-well plate were treated with IC_{50} concentrations of compounds **4l** and **4h** for 12 h. The control and treated cells were harvested, washed once with PBS and were lysed on ice for 10 min in lysis buffer containing 20 mM PIPES, 10 mM KCl, 2 mM $MgCl_2$, 4 mM DTT, 2 mM EDTA and 1 mM EGTA freshly supplemented with protease inhibitors. After centrifuging for 15 min (10,000 × *g*) cytosol fraction was transferred to fresh tubes and put on ice for performing the assays. Protein concentrations were determined by the Bradford method [32] using bovine serum albumin (BSA) as the standard. One hundred micrograms protein was used for each assay in the presence of 200 μM colorimetric caspase substrate (Ac-DEVD-*p*NA, Sigma) in 100 μL reaction buffer (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 0.1% CHAPS, 0.1 mM EDTA, 10% Glycerol). The absorbance was measured kinetically every 60 s for 120 min at 405 nm in an ELISA reader. Caspase-3 activity was expressed as the change of the activity in treated cancer cells compared to the untreated controls.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.09.017.

References

[1] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward, D. Forman, CA Cancer J. Clin. 61 (2011) 69–90.
[2] Cancer: WHO Factsheet No. 297, February 2014.
[3] National Vital Statistics Reports, vol. 61 (4), May 2013.
[4] M.M. Gottesman, Annu. Rev. Med. 53 (2002) 615–627.
[5] S.W. Fesik, Nat. Rev. Cancer 5 (2005) 876–885.
[6] R. Gerl, D.L. Vaux, Carcinogenesis 26 (2005) 263–270.
[7] E. Solary, L. Dubrez, B. Eymin, Eur. Respir. J. 9 (1996) 1293–1305.
[8] B. Favaloro, N. Allocati, V. Graziano, C. Di Ilio, V. De Laurenzi, Aging (Albany NY) 4 (2012) 330–349.
[9] S. Fulda, Int. J. Cancer 124 (2009) 511–515.
[10] J.C. Reed, J. Clin. Oncol. 17 (1999) 2941–2953.
[11] D.V. Krysko, T. Vanden Berghe, E. Parthoens, K. D'Herde, P. Vandenabeele, Methods Enzymol. 442 (2008) 307–341.
[12] J.C. Timmer, G.S. Salvesen, Cell Death Differ. 14 (2007) 66–72.
[13] C. Pop, G. Salvesen, S. Human, J. Biol. Chem. 284 (2009) 21777–21781.
[14] T. Kuno, T. Tsukamoto, A. Hara, T. Tanaka, J. Biophys. Chem. 3 (2012) 156–173.
[15] C.C. Chou, J.S. Yang, H.F. Lu, S.W. Ip, C. Lo, C.C. Wu, J.P. Lin, N.Y. Tang, J.G. Chung, M.J. Chou, Y.H. Teng, D.R. Chen, Arch. Pharm. Res. 33 (2010) 1181–1191.
[16] W. Kemnitzer, N. Sirisoma, B. Nguyen, S. Jiang, S. Kasibhatla, C. Crogan-Grundy, B. Tseng, J. Drewe, S.X. Cai, Bioorg. Med. Chem. Lett. 18 (2008) 6259–6264.
[17] W. Kemnitzer, N. Sirisoma, B. Nguyen, S. Jiang, S. Kasibhatla, C. Crogan-Grundy, B. Tseng, J. Drewe, S.X. Cai, Bioorg. Med. Chem. Lett. 19 (2009) 3045–3049.
[18] J.F. Kerr, C.M. Winterford, B.V. Harmon, Cancer 73 (1994) 2013–2026.
[19] S. Kaap, I. Quentin, D. Tamiru, M. Shaheen, K. Eger, H. Steinfelder, J. Biochem. Pharmacol. 65 (2003) 603–610.
[20] H. Piotrowska, K. Myszkowski, A. Ziółkowska, K. Kulcenty, M. Wierzchowski, M. Kaczmarek, M. Murias, E. Kwiatkowska-Borowczyk, J. Jodynis-Liebert, Toxicol. Appl. Pharmacol. 263 (2012) 53–60.
[21] M.A. Reddy, N. Jain, D. Yada, C. Kishore, V.J. Reddy, P.S. Reddy, A. Addlagatta, S.V. Kalivendi, B. Sreedhar, J. Med. Chem. 54 (2011) 6751–6760.
[22] M. Safavi, N. Esmati, S. Kabudanian Ardestani, S. Emami, S. Ajdari, J. Davoodi, A. Shafiee, A. Foroumadi, Eur. J. Med. Chem. 58 (2012) 573–580.
[23] (a) M. Mahmoodi, S. Emami, M. Safavi, S. Rajabalian, M.A. Mohagheghi, A. Khoshzaban, A. Samzadeh-Kermani, N. Lamei, A. Shafiee, A. Foroumadi, Arch. Pharm. Chem. Life Sci. 343 (2010) 411–416;
(b) H. Aryapour, G.H. Riazi, S. Ahmadian, A. Foroumadi, M. Mahdavi, S. Emami, Pharm. Biol. 50 (2012) 1551–1560.
[24] (a) H.A. Abdel-Aziz, T. Elsaman, A. Al-Dhfyan, M.I. Attia, K.A. Al-Rashood, A.M. Al-Obaid, Eur. J. Med. Chem. 70 (2013) 358–363;
(b) M.M. Kandeel, A.M. Kamal, E.K.A. Abdelall, H.A.H. Elshemy, Eur. J. Med. Chem. 59 (2013) 183–193.
[25] A. Fierro, M.C. Rezande, S. Sepulveda-Boza, M. Reyes-Parada, B.K. Cassels, J. Chem. Res. (S) 7 (2001) 294–296.
[26] M.C. Yan, Y.J. Jang, C.F. Yao, Tetrahedron Lett. 42 (2001) 2717–2721.
[27] O.O. Fadeyi, R.N. Daniels, S.M. DeGuire, C. Lindsley, Tetrahedron Lett. 50 (2009) 3084–3087.
[28] T. Mosmann, J. Immunol. Methods 65 (1983) 55–63.
[29] D.A. Scudiero, R.H. Shoemaker, K.D. Paull, A. Monks, S. Tierney, T.H. Nofziger, M.J. Currens, D. Seniff, M.R. Boyd, Cancer Res. 48 (1988) 4827–4833.
[30] D. Ribble, N.B. Goldstein, D.A. Norris, Y.G. Shellman, BMC Biotechnol. 5 (2005) 12.
[31] V. Gurtu, S.R. Kain, G. Zhang, Anal. Biochem. 251 (1997) 98–102.
[32] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.