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Synthesis and *in vitro* evaluation of 3-(4-nitrophenyl)coumarin derivatives in tumor cell lines

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ABSTRACT

Coumarins are naturally-occurring compounds that have attracted considerable interest due to their numerous biological activities depending on their pattern of substitution on the coumarin molecule. In this present investigation, we synthesized 3-(4-nitrophenyl)coumarin derivatives (**9a–e**) and evaluated their *in vitro* cytotoxic effect on human lung (A549), breast (MDA-MB-231) and prostate (PC3) cancer cell lines for 48 h using crystal violet dye binding assay. Cytotoxic effects of the most active compound on normal human lung (MRC-9) and breast (MCF-10A) cell lines, cell cycle analysis using flow cytometry and mitochondrial membrane potential (MMP) using Tetramethyl Rhodamine Methyl Ester (TMRM; rhodamine-123) fluorescent dye were also examined. Among the compounds that were evaluated, **9c** showed cytotoxic effect (active), caused significant cells arrest ($p < 0.05$) in G_0/G_1 and S phases of cell cycle and loss of MMP in A459, MDA-MB-231 and PC3 cell lines. Additionally, the cytotoxic effect of **9c** was compared to reference drugs (Coumarin and Docetaxel) for comparative study. These results further demonstrate that acetoxy group at C-7 and C-8 positions of **9c** are responsible for the observed cytotoxic effect in these cancer cell lines.

1. Introduction

Coumarins are important class of naturally-occurring heterocyclic compounds known as benzo-α-pyrones, a pyrone ring fused with a benzene nucleus [1,2]. Most coumarin compounds occurred as secondary metabolites in green plants, while some are produced by fungi and bacteria [3]. They exhibit diverse array of pharmacological and biochemical properties such as anti-coagulant [4], anti-tumor [5,6], anti-thrombotic [7], anti-viral [8,9], anti-bacterial [10], anti-microbial [11], anti-inflammatory [12,13] and anti-oxidant [14,15] properties. However, the anti-proliferative and anti-tumor activities of various coumarins have been extensively reported, e.g. simplest coumarin (**1**) and its metabolite, 7-hydroxycoumarin (**2**) have been reported to inhibit cell growth proliferation in various human cancer cell lines, such as A549 (lung), ACHN (renal), H727 (lung), MCF-7 (breast) and HL-60 (leukemia) [16–19]. They have also been reported in clinical trials to demonstrate activity against breast cancer, prostate cancer, malignant melanoma, and metastatic renal cell carcinoma [5,20,21]. In addition to their biological activities, coumarins are used as additives in food and cosmetics, and as optical brightening agents [22,23]. These properties of coumarins have led to considerable interest in their synthesis and possible applications as therapeutic agents for the treatment of various diseases.

Recent studies have shown that therapeutic applications, including pharmacological and biochemical properties of coumarins depends on the nature of the group present and its pattern of substitution on the core coumarin molecule [24,25]. For examples, acetoxycoumarin such as 7,8-diacetoxy-4-methylcoumarin (DAMC; **3**) exhibits anti-cancer and pro-oxidant activities in human tumor cell lines [26,27]; nitrocoumarins exhibit potent anti-microbial, anti-allergic and anti-cancer properties [28–30]; hydroxycoumarins exhibit diverse biological properties such as anti-coagulants, anti-arthritis, anti-inflammatory, anti-pyretic, anti-bacterial, anti-viral, and anti-cancer properties [5,31,32]; and 3-arylcoumarins commonly produced by plants have shown different types of biological properties such as antioxidant, anti-inflammatory, anticancer, anti-HIV and antimicrobial activities [33–36]. As part of our on-going investigation on coumarins as anticancer agents, we recently reported that 3-arylcoumarin containing acetoxy group (**4**) and basic amino side chain (**5** and **6**) exhibits cytotoxic activity in various cancer cell lines [37,38]. These studies and others not mentioned here strongly support the

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potential therapeutic applications of coumarin and its derivatives, making them attractive for further evaluation as novel therapeutic agents for cancer treatment.

Recent advances in anti-cancer studies have shown that heterocyclic compound containing *p*-nitrophenyl pharmacophore offers great potential for the development of novel anti-cancer agents [39,40]. This finding aroused our interest in the presence cytotoxicity studies of coumarins containing *p*-nitrophenyl groups substituted at the 3-position with the aim of obtaining more potent biologically active coumarins. Therefore, we herein report the synthesis of 3-(4-nitrophenyl)coumarin derivatives (**9a–e**, Table 1); and evaluation of their *in vitro* cytotoxic effect on A549, MDA-MB-231 and PC3 cancer cell lines. In addition, cytotoxic effect of the most active compound on normal (MRC-9 and MCF-10A) cell lines, cell cycle and MMP were also evaluated. Finally, cytotoxic effect of the most active compound was also compared to Coumarin and Docetaxel drugs.

2. Materials and methods

2.1. Chemistry

2.1.1. Materials

Commercial grade solvents and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Alfa Aeser (Ward Hill, MA, USA) and used without further purification. The NMR spectra were recorded on Varian 300 MHz spectrometer (^{1}H: 300 MHz, ^{13}C: 75 MHz,). The appropriate deuterated solvents are indicated in the experimental procedure and line positions recorded in (δ) *ppm* from the reference signal. ESI-TOF Mass Spectrometer was recorded on Finnigan LCQ – Quadrupole Ion Trap (Thermo Finnigan, San Jose, CA), and the HPLC pump was an Agilent (HP) 1100 series pump or Applied Biosystems model 400 pump. CHN elemental analysis was performed by combustion using automatic analyzers. To estimate the degree of purity of the active compound (**9c**), HPLC analysis was conducted with Waters Alliance 2996 HPLC system equipped with Wasters 2996 Photodiode array detector. HPLC grade acetonitrile and HPLC water (Merck) was used for the chromatography. Melting points were determined on a Gallenkamp (UK) apparatus.

2.1.2. General experimental procedures

To a mixture of 2-hydroxybenzaldehyde (**7a**) [or 2-hydroxyacetophenone (**7b**), 2,3,4-trihydroxy benzaldehyde (**7c**), 2-hydroxy-4-methoxybenzophenone (**7d**) or 2-hydroxy- benzophenone (**7e**)] (7.4 mmol) and 4-nitrophenylacetic acid (**8**) (8 mmol) in acetic anhydride (3 ml) was added triethylamine (99%, 1 ml). The reaction mixture was then refluxed overnight at 90 °C, allowed to cool to r.t. and 10 ml of ether was added with occasional stirring for another 2–3 h to afford the desired product as a precipitate that was collected using vacuum filtration.

The synthesis and structural characterization of **9a** has been previously reported in literature [41–43].

*2.1.2.1. 3-(4-Nitrophenyl)coumarin (**9a**).* This title compound was prepared according to the above experimental procedure starting from 2-hydroxybenzaldehyde (**7a**) to afford **9a** as a yellow precipitate (1.02 g, 52%) with m.p. = 267.2–268.4 °C (Literature reported m.p. = 267–268 °C). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.33–7.43 (2H, ArH), 7.59–7.65 (2H, ArH), 7.91–7.96 (3H, ArH) and 8.30–8.33(2H, ArH); HRMS (ESI) (m/z): for $C_{15}H_9NO_4$ calcd: 268.0604; [M + H]; found: 268.0605.

*2.1.2.2. 4-Methyl-3-(4-nitrophenyl)coumarin (**9b**).* This title compound was prepared according to the above experimental procedure starting from 2-hydroxyacetophenone (**7b**) to afford **9b** as a yellow precipitate (1.52 g, 77%) with m.p. = 200.6–201.8 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.36 (3H, s. CH_3), 7.35–7.41 (2H, ArH), 7.52–7.55 (2H, ArH), 7.58–7.64 (1H, ArH), 7.72–7.45 (1H, ArH) and 8.30–8.33 (2H, ArH). ^{13}C-NMR (75 MHz, $CDCl_3$) 16.73 (CH_3), 11.7.03, 120.00, 123.63, 124.70, 125.21, 125.35, 131.43, 132.23, 141.35, 147.55, 148.96, 152.74 (Ar—C) and 160.25 (C═O); HRMS (ESI) (m/z): for $C_{16}H_{11}NO_4$ calcd: 282.0768; [M + H]; found: 282.0767.

*2.1.2.3. 7,8-Diacetoxy-3-(4-nitrophenyl)coumarin (**9c**).* This title compound was prepared according to the above experimental procedure starting from 2,3,4-Trihydroxy benzaldehyde (**7c**) to afford **9c** as a yellow precipitate (2.66 g, 97%) with m.p. = 224.4–226.1 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.37–245 (6H, s, 2 × OCOCH_3), 7.17–7.20 (1H, ArH), 7.46–7.49 (1H, ArH), 7.85–7.92 (3H, ArH) and 8.28–8.30 (2H, ArH). ^{13}C-NMR (75 MHz, $CDCl_3$) 20.34 and 20.68 (2 × $OCOCH_3$), 117.88, 119.62, 123.70, 125.49, 125.83, 129.45, 140.56, 146.00, 145.85, 146.86, 147.87, 158.39 (Ar—C), and 167.33 and 167.72 (C═O); HRMS (ESI) (m/z): for $C_{19}H_{13}NO_8$ calcd: 384.0714; [M + H]; found: 384.0716. Anal. Calcd for $C_{19}H_{13}NO_8$: C, 59.54; H, 3.42; N, 3.65. Found: C, 59.70; H, 3.41; N, 3.69. Purity (HPLC): 99.17%.

*2.1.2.4. 7-Methoxy-3-(4-nitrophenyl)-4-phenylcoumarin (**9d**).* This title compound was prepared according to the above experimental

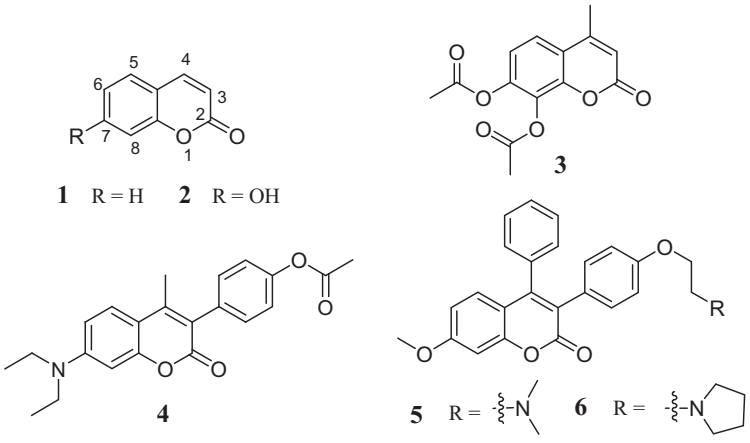


Fig. 1. Structures of (**1**) Coumarin, (**2**) 7-hydroxycoumarin. (**3**) 7,8-diacetoxy-4-methyl coumarin (DAMC), (**4**) 4-(7-(diethylamino)-4-methyl-2-oxo-2H-chromen-3-yl)phenyl acetate (**5**) 3-(4-(2-(dimethylamino)ethoxy)phenyl)-7-methoxy-4-phenylcoumarin (**6**) 3-(4-(2-(pyrrodin-1-yl)- ethoxy)phenyl)-7-methoxy-4-phenylcoumarin.

procedure starting from 2-hydroxy-4-methoxybenzophenone (**7d**) to afford **9d** as a yellow precipitate (1.45 g, 52%) with m.p. = 214.9–216.6 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 3.91 (3H, s. OCH_3), 6.78–6.82 (1H, ArH), 6.94–6.95 (1H, ArH), 7.01–7.17 (3H, ArH), 7.27–7.36 (5H, ArH) and 8.01–8.06 (2H, ArH). ^{13}C-NMR (75 MHz, $CDCl_3$) 55.91 (OCH_3), 100.69, 112.78, 113.50, 121.41, 132.91, 128.66, 128.99, 129.16, 131.90, 133.93, 141.32, 146.72, 153.28, 155.21, 160.86 (Ar—C) and 163.22 (C═O); HRMS (ESI) (m/z): for $C_{22}H_{15}NO_5$ calcd: 374.1023; [M + H]; found: 374.1035.

*2.1.2.5. 3-(4-Nitrophenyl)-4-phenyl-coumarin (**9e**).* This title compound was prepared according to the above experimental procedure starting from 2-hydroxybenzophenone (**7e**) to afford **9e** as a yellow precipitate (1.43 g, 56%) with m.p. = 236.1–239.0 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.11–7.14 (2H, ArH), 7.24–7.28 (2H, ArH), 7.31–7.37 (5H, ArH), 7.45–7.48 (1H, ArH), 7.58–7.63 (1H, ArH) and 8.04–8.07(2H, ArH). ^{13}C-NMR (75 MHz, $CDCl_3$) 116.97, 119.99, 122.97, 124.56, 124.80, 128.10, 128.72, 129.10, 129.18, 131.77, 132.41, 133.60, 140.97, 146.92, 153.08, 153.38 (Ar—C) and 160.49 (C═O); HRMS (ESI) (m/z): for $C_{21}H_{13}NO_4$ calcd: 344.0917; [M + H]; found: 344.0927).

2.2. Biology

2.2.1. Chemicals

F12 K medium, RPMI medium, penicillin–streptomycin antibiotic solution (100×), fetal bovine serum (FBS), Trypsin–EDTA solution (1×), phosphate buffer (PBS), 50% glutaraldehyde, crystal violet, propidium iodide, Rhodamine-123, IGPAL CA-630, Coumarin, Docetaxel and RNase were obtained from Sigma–Aldrich Company (St. Louis, MO, USA). The potassium phosphate, EDTA, D-glucose, ethanol were obtained from Thomas Scientific Company (Swedesboro, NJ, USA).

2.2.2. Cell line maintenance

The cell lines (A549, MRC-9, MDA-MB-231, MCF-10A and PC3) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured as per the guidelines supplied. The cells were maintained in F12 K (A549, PC3) or MEM (MRC-9) or RPMI (MDA-MB-231) or DMEM-F12 (MCF-10A) medium containing 100 units of penicillin/ml, 100 μg of streptomycin/ml, 2 mM L-glutamine and 10% FBS in T-75 cm^2 flasks at 37 °C in a 5% CO_2 incubator.

2.2.3. Treatment of cells

The cells (A549, MRC-9, MDA-MB-231, MCF-10A or PC3) were plated at a density of 5×10^4 cells per well in polystyrene, flat bottom 24-well microtiter plates (Corning Costar, Rochester, NY, USA) in complete medium containing 10% FBS and allowed to stabilize overnight in a CO_2 incubator at 37 °C. Next, the cells were treated with compounds (**9a–e**) at different concentrations (0, 10, 25, 50, 75 and 100 μM) in a final volume of 1 ml per well in triplicate wells for each treatment for 48 h at 37 °C in a 5% CO_2 incubator. All studies were repeated at least twice.

2.2.4. Evaluation of cell viability

At the end of incubation period, the cell viability was evaluated by dye uptake assay according to previous reported method by Badisa et al. [44]. Glutaraldehyde (400 μl of 0.25%) was added to each well and incubated for 30 min at room temperature to fix the cells. The glutaraldehyde (0.07% final concentration in the well) in the crystal violet dye staining assay procedure fixed the viable cells after the treatment with compounds. The plates were rinsed with water to wash off the dead cells and dried under airflow inside a laminar hood for 5–10 min. Crystal violet (400 μl of 0.1%) was added to each well, incubated for 15 min, washed and dried. To solubilize the dye, 1 ml of 0.05 M sodium phosphate solution (monobasic) in 50% ethyl alcohol was added to each well and the plates were read at 540 nm in a plate reader (Bio-Tek EL800 Plate Reader). The mean absorbance value of control was considered as 100% and the treated sample percentages were calculated by comparing the treated samples absorbance with the mean absorbance of control.

2.2.5. Cell cycle analysis

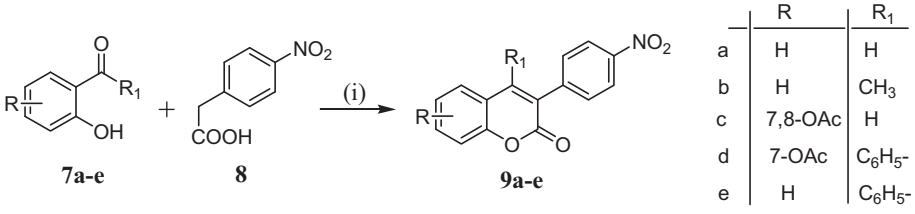
The cell cycle analysis was evaluated using Accuri flow cytometer (BD Biosciences, San Jose, CA). Cells (A549, MDA-MB-231 and PC3) at a density of 0.65×10^6 cells per T-25 cm^2 flask were plated and incubated overnight. The cells were then treated with 0, 10 and 20 μM of the active compound in triplicate flasks and incubated for 48 h in a 5% CO_2 incubator at 37 °C. At the end of incubation, the cells were trypsinized and centrifuged at 2500 rpm for 10 min at room temperature. The pellet was suspended in 1 ml of PBS and transferred to Eppendorff tube. The cells were pelleted by centrifugation at 2500 rpm for 5 min. Then the cells were stained with 500 μl of Vindelov's reagent containing 0.01 mg/ml of ribonunuclease A and 0.075 mg/ml of propidium iodide and 1 μl/ml of IGPAL CA-630 in phosphate buffered saline for overnight at 4 °C in dark. The distribution of cells in each phase was analyzed with the Accuri flow cytometer (Becton Dickinson, San Jose, CA). In each sample, a total of 50,000 events were analyzed separately. The Accuri C6 software was used for acquisition and analysis of the data and the percentage of cells in each phase.

2.2.6. Measurement of MMP

The loss of MMP was evaluated using rhodamine-123 fluorescent dye in A549, MDA-MB-231 and PC3 cells. At the end of incubation, cells were fixed with 400 μl of 0.25% aqueous glutaraldehyde containing rhodamine-123 to yield a final concentration of 1 μM for 30 min at room temperature. The supernatant was discarded, and the plates were washed with tap water and air dried in the hood. Finally, 500 μl of 0.1% Triton X 100 in dPBS was added per well and incubated at 37 °C for 1 h. The plates were read with the excitation filter set at 485 nm and the emission filter at 538 nm in TECAN microplate Fluorometer.

2.3. Statistical analysis

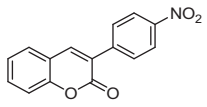
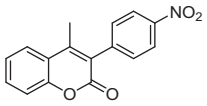
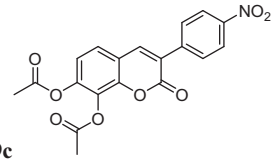
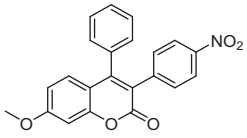
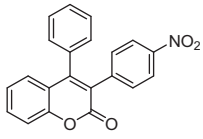
The viability values were presented as mean ± standard deviation (n = 3). All treated cells data were presented as percentage values in comparison to the untreated control (100%). The via-



	R	R_1
a	H	H
b	H	CH_3
c	7,8-OAc	H
d	7-OAc	C_6H_5-
e	H	C_6H_5-

Scheme 1. Reagents and Conditions: (i) AC_2O, NEt_3, refluxed overnight at 90 °C.

Table 1
The CC_{50} values for compounds **9a–e** and reference drugs (Coumarin and Docetaxel) tested on lung (A549), breast (MDA-MD-231) and prostate (PC3) cancer cell lines for 48 h.

	CC_{50} (μM) mean ± SD				
Compounds	A549	MRC-9	MDA-MB-231	MCF-10A	PC-3
9a 	>100.0	ND	>100.0	ND	>100.0
9b 	>100.0	ND	>100.0	ND	>100.0
9c 	12.2 ± 1.85	17.1 μM ± 1.07	27.6 ± 3.39	9.6 μM ± 1.41	18.2 ± 0.15
9d 	>100.0	ND	>100.0	ND	>100.0
9e 	>100.0	ND	>100.0	ND	>100.0
Docetaxel	9.40 ± 0.07	ND	7.30 ± 0.03	ND	9.44 ± 0.44
Coumarin	>100	ND	>100	ND	>100

Data represent the average of two values ($n = 6$). The cytotoxic concentration (CC_{50}) value was determined from the graph where the live and dead cells line graphs meet in the Graph pad Prism. Drugs effects were determined for 48 h exposure. ND: Not Determined.

bility and CC_{50} graphs were plotted in Prizm 3.00 software (GraphPad Software, Inc., San Diego, CA, USA). The CC_{50} value was calculated from the CC_{50} graph where the two lines meet (the dose at which 50% of the cells dies) using Prizm 5 software.

3. Result

3.1. Synthesis of 3-(4-nitrophenyl)coumarin derivatives (*9a–e*)

In this present investigation, we reported the synthesis of 3-(4-nitrophenyl)coumarin derivatives (**9a–e)** *via* modified reported based-catalyzed Perkin condensation reaction [45–47] (Scheme 1). This process involved refluxing a mixture of benzaldehyde (acetophenone or benzophenone) and 4-nitrophenylacetic acid in the presence of triethylamine in acetic anhydride overnight at 90 °C to afford the desired compounds as solid between 52% and 97% yields. The synthesized compounds were characterized using proton (^1H) and carbon (^{13}C) NMR, HRMS and CHN elemental analyses as indicated in the experimental section. The HPLC analysis was also conducted to estimate the degree of purity of the active compound (**9c**) and result indicated 99.17% purity.

3.2. Cytotoxicity studies

The synthesized compounds **(9a–e)** were evaluated for their *in vitro* cytotoxic effect using crystal violet dye-staining assay. The cytotoxic concentration values (CC_{50}, the concentration of tested drug where 50% dead cells is observed in the treated sample compared to the untreated control) for **9a–e**, Coumarin and Docetaxel were calculated according to the Ipsen' method [48] and are given in Table 1. Result indicated that **9c** showed cytotoxic effect in A549 (CC_{50} = 12.2 μM), MDA-MD-231 (CC_{50} = 27.6 μM) and PC3 (CC_{50} = 18.2 μM) cancer cell lines, while **9a**, **9b**, **9d** and **9e** did not show cytotoxic effect (CC_{50} > 100 μM; inactive). The compound **9c** was most cytotoxic to A549 cells, causing dose-dependent cell death in comparison to the untreated control cells (Fig. 2). Comparison of the cytotoxic effect of **9c** with Coumarin and Docetaxel revealed that (1) Coumarin was not active in the three cancer cell lines (CC_{50} > 100) and (2) **9c** showed comparable cytotoxicity to Docetaxel on A549 cell line and lesser cytotoxicity compared to

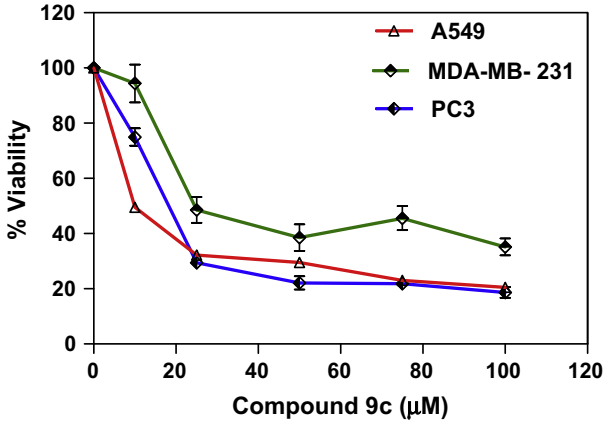


Fig. 2. Effect of compound **9c** on A549, MDA-MB-231 and PC3 cell viability. Data are represented as the mean and SD, $n = 6$.

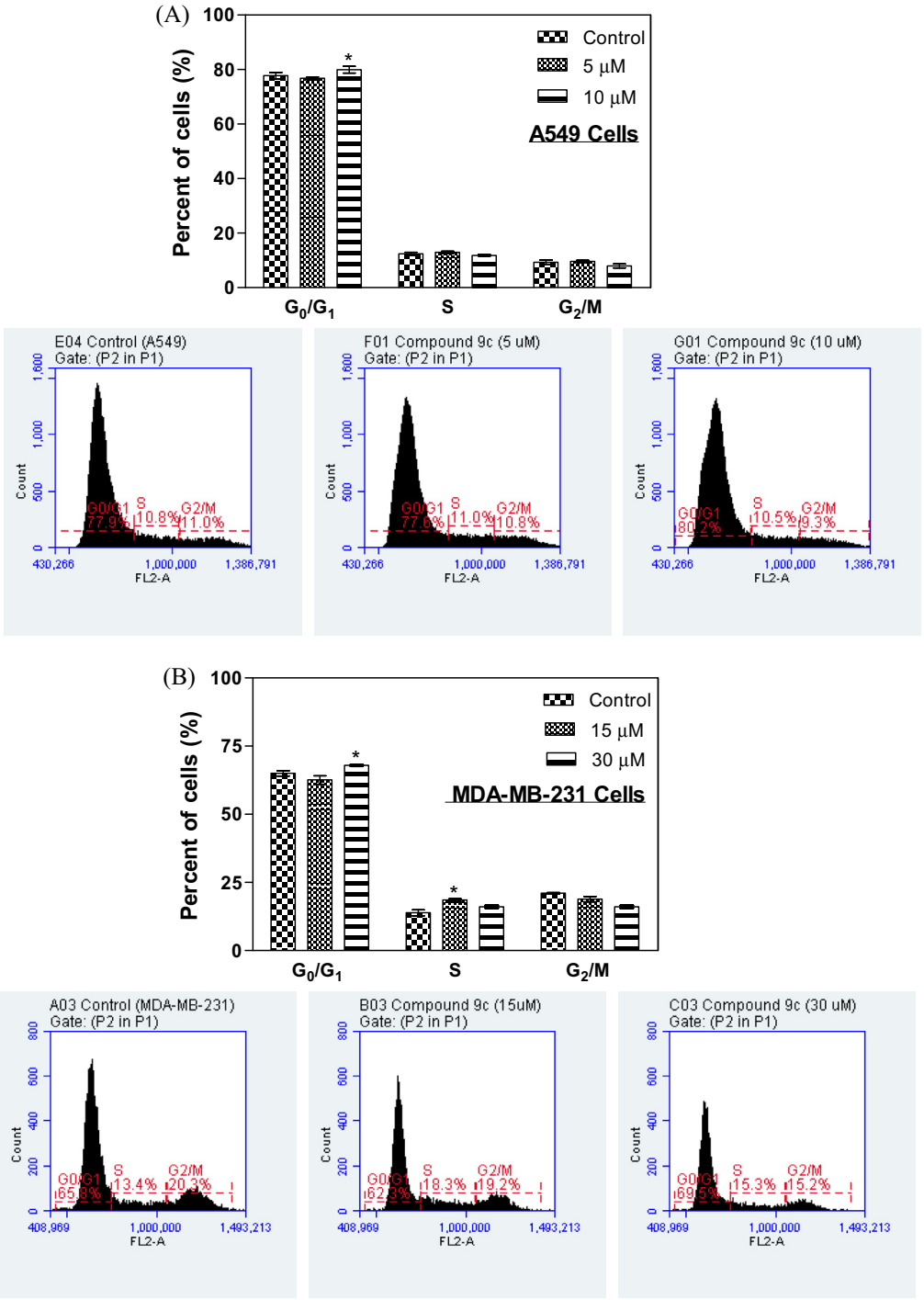


Fig. 3. Effect of compound **9c** on (A) A549, (B) MDA-MB-231 and (C) PC3 cell cycle. Data are represented as mean and SD, $n = 3$. *Statistically significant difference from the control ($p < 0.05$) using Dunnett's multiple comparison test.

Docetaxel on both MDA-MD-231 and PC3 cell lines (Table 1). Additionally, **9c** showed similar cytotoxic effect on both cancer (A549 and MDA-MB-231) and normal (MRC-9 and MCF-10A) cell lines (Table 1).

3.3. Cell cycle analysis

Cells (A549, MDA-MB-231 and PC3) were treated with **9c** for 48 h, followed by staining with propidium iodide. The percentage of cells in G_1, G_2/M and S phases of the cell cycle was analyzed using a flow cytometer. Result indicated that treatment of A549, MDA-MB-231 and PC3 cells with **9c** caused cell cycle arrest ($p < 0.05$) in concentration dependent manner (Fig. 3A–C). Furthermore, it was observed that **9c** arrest (i) A549 cells in G_0/G_1 phase at 10 μM concentration (2.17%) (Fig. 3A), (ii) MDA-MB-231 cells in G_0/G_1 phase at 30 μM concentration (2.90%), and S phase at both 15 μM concentration (4.72%) and 30 μM concentration (2.23%) (Fig. 3B) and (iii) PC3 cells in G_0/G_1 phase at both 10 μM concentration (3.04%) and 20 μM concentration (2.84%) with respect to the untreated control cells (Fig. 3C).

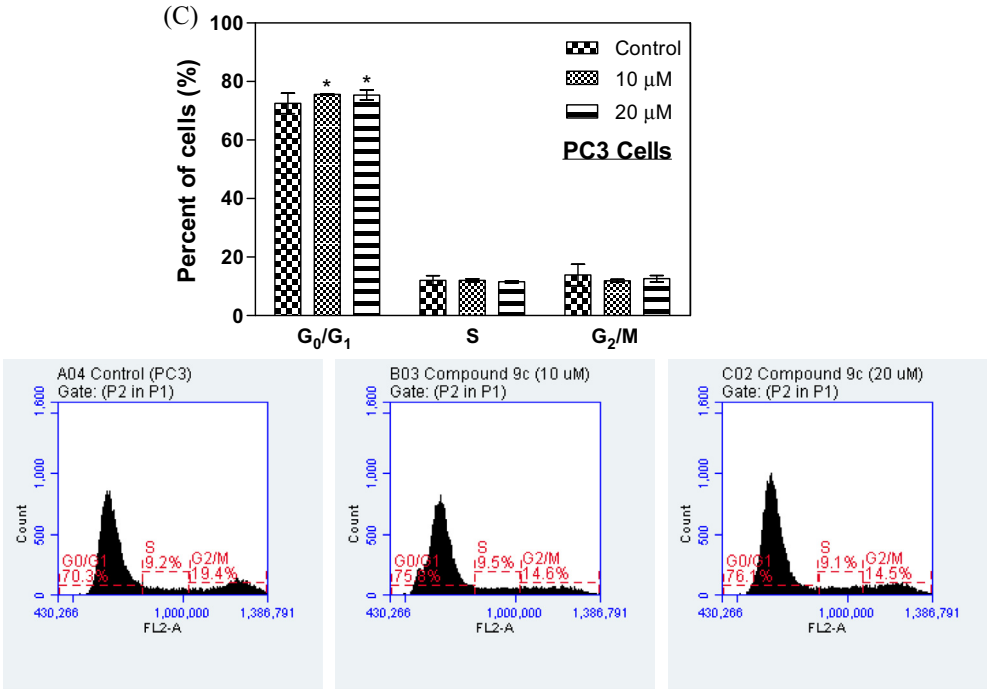


Fig. 3 (*continued*)

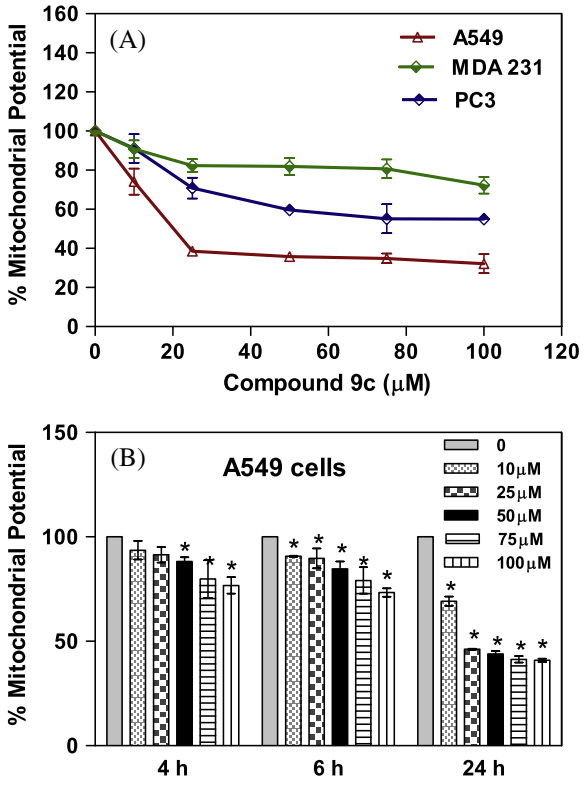


Fig. 4. Effect of compound **9c** on mitochondrial membrane potential in (A) A549, MDA-MB-231 and PC3 cell lines for 48 h and (B) A549 cell lines for 4, 6, 24 h. Data are represented as mean and SD, $n = 3$. *Statistically significant difference from the control ($p < 0.05$) using Dunnett's multiple comparison test.

3.4. Measurement of MMP

Cells (A549, MDA-MB-231 and PC3) were treated with **9c** for 48 h and then fixed with rhodamine-123 dye. Results indicated that **9c** caused loss of MMP in the following order; A549 (lung) > PC 3 (prostate) > MDA-MB-231 (breast) cells (Fig. 4A). The percentage of MMP in comparison to the untreated control cells (100%) in (i) A549 cells at 10 μM (74.2% ± 3.86), 25 μM (38.7% ± 0.99), 50 μM (35.8% ± 1.21), 75 μM (34.9% ± 1.45) and 100 μM (32.2% ± 2.82); (ii) MDA 231 cells at 10 μM (90.8% ± 2.56), 25 μM (82.4% ± 1.87), 50 μM (81.9% ± 2.52), 75 μM (80.7% ± 2.772) and 100 μM (72.4% ± 2.47) and (iii) PC3 cells at 10 μM (91.1% ± 4.22), 25 μM (70.9% ± 3.07), 50 μM (59.6% ± 0.66), 75 μM (55.2% ± 4.29) and 100 μM (55.0% ± 0.66); indicating loss of MMP respectively. Additionally, cells exposed to **9c** treatment at shorter period of time (4, 6 and 24 h) also showed dose and time dependent loss of MMP in all three cancer cell lines as indicated in Fig. 4B.

4. Discussion

Interest in the synthesis and biological evaluation of coumarins has increased over the years due to their diverse pharmaceutical activities. As part of our ongoing investigation involving coumarins aims at evaluating the role of substituent effect in modulating cytotoxicity, we synthesized series of 3-(4-nitrophenyl)coumarin derivatives (**9a–e**, Table 1) and evaluated their cytotoxic effect on A549, MDA-MB-231 and PC3 cancer cell lines. The *in vitro* cytotoxicity results indicate that **9c** bearing acetoxy group at C-7 and C-8 positions on the benzenoid ring of coumarin molecule showed significant cytotoxic effect with respect to untreated cells, while absence of this group as indicated in **9a**, **9b**, **9d** and **9e** resulted in loss of cytotoxic activity (Table 1). This finding is supported by previous reports indicating that the presence of acetoxy group at C-7 and C-8 positions of the coumarin molecule (**3**) enhanced drug activity such as anti-cancer, antioxidant and radicals scavenging properties [26,27,49]. Interestingly, Coumarin was relatively non-toxic in all tested cancer cell lines; indicating that it is cytotoxic effect was metabolism and dose-time dependent [50,51]. Additionally, compound **9c** showed similar cytotoxicity on A549, and two-fold (PC3) and fourth-fold (MDA-MB-231) decreased in cytotoxicity compared to Docetaxel. Docetaxel (formerly called taxotere) belongs to a new class of cytotoxic agents known as tax-

anes and is used in the treatment of breast cancer, lung cancer, prostate cancer, stomach cancer, and head/neck cancer [52]. Comparison of **9a** and **9c** based on structural relationship (SAR) activity indicates that presence of *p*-nitrophenyl group at the C-3 position did not make any contribution to the observed cytotoxic effect of **9c**; further supporting the beneficial effect of acetoxy group at C-7 and C-8 positions on the coumarin molecule in modulating cytotoxicity. The order of **9c** cytotoxic effect based on CC_{50} values is A549 > PC3 > MDA-MD 231 cells. However, the non-selectivity of **9c** in normal MRC-9 (CC_{50} = 17.1 μM ± 1.07) and MCF-10A (CC_{50} = 9.6 μM ± 1.41) cell lines is of utmost concern, since one of the important criteria in the development of therapeutic drugs for cancer treatment is to have few or no side-effects on normal cells of patients undergoing chemotherapy. A possible reason for the lack of 9c selective cytotoxicity could be due that the two selected non-malignant lines are female-origin cells [53].

One effective strategy for controlling tumor growth is the inhibition of cell cycle progression in cancer cells [54]. Previous studies have demonstrated that toxic effect of compound on different phases of the cell cycle can activate or inhibit of cell growth and proliferation of cancer cells [55,56]. In the present investigation, it was observed that **9c** caused cell arrest of (i) A549, MDA-MB-231 and PC3 cells in G_0/G_1 phase, indicating apoptotic mode of cell death and (ii) MDA-MB 231 cells in S phase, indicating inhibition of DNA synthesis (Fig. 3). This finding is consistent with previous investigations indicating that coumarins caused G_0/G_1 and S phases cell arrest in different cancer cell lines [57,58].

Rhodamine-123, a green-fluorescent dye, has been used to measure MMP changes in cells [59]. This dye is selectively taken up by mitochondria and the amount taken up by the cells is directly proportional to its membrane potential [59]. Thus, evaluation of MMP changes in living cells is of critical important in accessing the effect of chemicals on mitochondria function [60]. The present investigation indicates loss of MMP in the following order: MDA-MB-231 > PC3 > A549 cell lines after 48 h treatment with **9c** (Fig. 4A). Furthermore, **9c** caused dose-time dependent loss of MMP in A549 cells during the early stages of the treatment; suggesting that it might be using intrinsic apoptotic pathway to inflict cell death (Fig. 4B) [61]. Interestingly, the observed correlation between cytotoxic effect and loss of MMP in these cancer cell lines (Figs. 1 and 4) indicates that the elicited differential cytotoxicity of **9c** may be associated with the loss of MMP.

5. Conclusion

A series of 3-(4-nitrophenyl)coumarin derivatives (**9a–e**) were synthesized, characterized and evaluated for their *in vitro* cytotoxic effect on A549, MDA-MB-231 and PC3 cancer cell lines. Compound **9c** bearing acetoxy group at C-7 and C-8 positions showed cytotoxic effect compared to other synthesized analogs (**9a**, **9b**, **9d** and **9e**). The cytotoxic effect of **9c** is associated with cell arrest in G_0/G_1 and S phases of the cell cycle, and loss of MMP. Additionally, the elicited differential cytotoxicity of **9c** is associated with the loss of MMP based on the observed correlation between cytotoxic effect and loss of MMP. This present investigation further correlates the importance of acetoxy group at C-7 and C-8 positions on the coumarin molecule with respect to modulating cytotoxic effect and thus indicates that **9c** could serve as an attractive leads in the future development of new cytotoxic compounds that are more potent and selective.

Declaration of interest statement

The authors declare that they have no financial or non-financial competing interests.

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References

[1] R.D.H. Murray, J. Mendez, S.A. Brown, The Natural Coumarins: Occurance Chemistry and Biochemistry, John Wiley, New York, 1982, pp. 282–289.
[2] F. Borges, F. Roleira, N. Milhazes, L. Santana, E. Uriarte, Curr. Med. Chem. 12 (8) (2005) 887–916.
[3] Z.E. Nazari, M. Iranshahi, Phytother. Res. 25 (2011) 315–323.
[4] I. Manolov, C. Maichle-Moessmer, N. Danchev, Eur. J. Med. Chem. 41 (2006) 882–890.
[5] I. Kostova, Curr. Med. Chem. Anticancer Agents 5 (2005) 29–46.
[6] H. Zhao, B. Yan, L.B. Peterson, B.S. Blagg, ACS Med. Chem. Lett. 3 (2012) 327–331.
[7] J.R. Hoult, M. Paya, Gen. Pharmacol. 27 (1996) 713–722.
[8] L. Xie, Y. Takeuchi, L.M. Cosentino, A.T. McPhail, K.H. Lee, J. Med. Chem. 44 (2001) 664–671.
[9] Z.M. Nofal, M.I. El-Zahar, S.S. AbdEl-Karim, Molecules 5 (2000) 99–113.
[10] P. Aragade, V. Maddi, S. Khode, M. Palkar, P. Ronad, S. Mamledesai, D. Satyanarayana, Arch. Pharm. (Weinheim) 342 (2009) 361–366.
[11] D.A. Ostrov, J.A. Hernandez Prada, P.E. Corsino, K.A. Finton, N. Le, T.C. Rowe, Antimicrob. Agents Chemother. 51 (2007) 3688–3698.
[12] S. Khode, V. Maddi, P. Aragade, M. Palkar, P.K. Ronad, S. Mamledesai, A.H. Thippeswamy, D. Satyanarayana, Eur. J. Med. Chem. 44 (2009) 1682–1688.
[13] I.E. Bylov, M.V. Vasylyev, Y.V. Bilokin, Eur. J. Med. Chem. 34 (1999) 997–1001.
[14] O.M. Singh, N.S. Devi, D.S. Thokchom, G.J. Sharma, Eur. J. Med. Chem. 45 (2010) 2250–2257.
[15] S. Stanchev, V. Hadjimitova, T. Traykov, T. Boyanov, I. Manolov, Eur. J. Med. Chem. 44 (2009) 3077–3082.
[16] M. Baba, Y. Jin, A. Mizuno, H. Suzuki, Y. Okada, N. Takasuka, H. Tokuda, H. Nishino, T. Okuyama, Biol. Pharm. Bull. 25 (2002) 244–246.
[17] D. Thornes, L. Daly, G. Lynch, H. Browne, A. Tanner, F. Keane, S. O'Loughlin, T. Corrigan, P. Daly, G. Edwards, J. Henley, T. Healy, F. Keane, F. Lennon, N. McMurray, S. O'Loughlin, M. Shine, A. Tanner, Eur. J. Surg. Oncol. 15 (1989) 431–435.
[18] S. Stanchev, G. Momekov, F. Jensen, I. Manolov, Eur. J. Med. Chem. 43 (2008) 694–706.
[19] M.E. Marshall, K. Kervin, C. Benefield, A. Umerani, S. Albainy-Jenei, Q. Zhaoand, M.B. Khazaeli, J. Cancer Res. Clin. Oncol. 120 (Suppl.) (1994) S3–S10.
[20] J.L. Mohler, L.G. Gomella, E.D. Crawford, L.M. Glode, C.D. Zippe, W.R. Fair, M.E. Marshall, Prostate 20 (1992) 123–131.
[21] A. Lacy, R. O'Kennedy, Curr. Pharm. Des. 10 (2004) 3797–3811.
[22] R. O'Kennedy, R.D. Thornes, Coumarins: Biology, Applications and Mode of Action, John Wiley & Sons, Chichester, 1997.
[23] D. Bogdal, J. Chem. Res. (S) (1998) 468–469.
[24] M.A. Musa, J.S. Cooperwood, M.O. Khan, Curr. Med. Chem. 15 (2008) 2664–2679.
[25] A. Carotti, A. Carrieri, S. Chimichi, M. Boccalini, B. Cosimelli, C. Gnerre, P.A. Carrupt, B. Testa, Bioorg. Med. Chem. Lett. 12 (2002) 3551–3555.
[26] L. Koshy, B.S. Dwarakanath, H.G. Raj, R. Chandra, T.L. Mathew, Indian J. Exp. Biol. 41 (2003) 1273–1278.
[27] P. Khurana, R. Kumari, P. Vohra, A. Kumar, Seema, G. Gupta, H.G. Raj, B.S. Dwarakanath, V.S. Parmar, D. Saluja, M. Bose, A. Vij, N.K. Chaudhary, J.S. Adhikari, Y.K. Tyagi, E. Kohli, Bioorg. Med. Chem. 14 (2006) 575–583.
[28] I. Fujii, Y. Mano, N. Hirayama, Anal. Sci. 22 (2006) 117–118.
[29] R.S.A. de Araújo, F.Q.S. Guerra, E.deO. Lima, C.A. de Simone, J.F. Tavares, L. Scotti, M.T. Scotti, T.M. de Aquino, R.O. de Moura, F.J.B. Mendonça Jr., J.M. Barbosa-Filho, Int. J. Mol. Sci. 14 (2013) 1293–1309.
[30] Ž. Debeljak, A. Škrbo, I. Jasprica, A. Mornar, V. Plečko, M. Banjanac, M. Medić-Šarić, J. Chem. Inf. Model. 47 (2007) 918–926.
[31] J.-C. Jung, O.-S. Park, Molecules 14 (2009) 4790–4803.
[32] S. Thaisrivongs, P.K. Tomich, K.D. Watenpaugh, K.T. Chong, W.J. Howe, C.P. Yang, J.W. Strohbach, S.R. Turner, J.P. McGrath, J. Med. Chem. 37 (1994) 3200–3204.
[33] S. Demizu, K. Kajiyama, K. Takahashi, Y. Hiraga, S. Yamamoto, Y. Tamura, K. Okada, T. Kinoshita, Chem. Pharm. Bull. 36 (1988) 3474–3479.
[34] H.R.J. El-Seedi, Nat. Prod. 70 (2007) 118–120.
[35] L.S. You, R. An, X.H. Wang, Y.M. Li, Bioorg. Med. Chem. Lett. 20 (2010) 7426–7428.
[36] D. Olmedo, R. Sancho, L.M. Bedoya, J.L. Lopez-Perez, E. Del Olmo, E. Munoz, J. Alcami, M.P. Gupta, A. San Feliciano, Molecules 17 (2012) 9245–9257.
[37] M.A. Musa, V.L. Badisa, L.M. Latinwo, J. Cooperwood, A. Sinclair, A. Abdullah, Anticancer Res. 31 (6) (2011) 2017–2022.
[38] M.A. Musa, V.L. Badisa, L.M. Latinwo, C. Waryoba, N. Ugochukwu, Anticancer Res. 30 (11) (2010) 4613–4617.
[39] S.S. Shah, G. Rivera, M. Ashfaq, Mini. Rev. Med. Chem. 13 (2013) 70–86.
[40] S. Ibsen, E. Zahavy, W. Wrasdilo, M. Berns, M. Chan, S. Esener, Pharm. Res. 27 (2010) 1848–1860.
[41] S.H. Mashraqui, D. Vashi, H.D. Mistry, Synth. Commun. 34 (2004) 3129–3134.
[42] M.J. Matos, S. Vazquez-Rodriguez, F. Borges, L. Santana, E. Uriarte, Tetrahedron Lett. 52 (2011) 1225–1227.
[43] C. Rondestvedt, S. Christian, O. Vogl, J. Am. Chem. Soc. 77 (1955) 3401–3402.

[44] R.B. Badisa, O. Tzakou, M. Couladis, E. Pilarinou, Phytother. Res. 17 (2003) 472–476.
[45] R.M. Kanojia, N. Jain, J. Xu, Z. Sui, Tetrahedron Lett. 45 (2004) 5837.
[46] A. Horvath, K. De Smet, D. Ormerod, D. Depré, C. Pérez-Balado, T. Govaerts, D. Van den Heuvel, I. Schorpion, I. Org. Process Res. Dev. 9 (2005) 356.
[47] M.A. Musa, A. Zhou, O.A. Sadik, Med. Chem. 7 (2011) 112–120.
[48] J. Ipsen, P. Feigl, Bancroft's Introduction to Biostatistics, second ed., Harper and Row, New York, 1970. pp. 164.
[49] H.G. Raj, V.S. Parmar, S.C. Jain, S. Goel, P. Himanshu, S. Malhotra, A. Singh, C.E. Olsen, J. Wengel, Bioorg. Med. Chem. 6 (1998) 833–839.
[50] M. Kawase, H. Sakagami, K. Hashimoto, S. Tani, H. Hauer, S.S. Chatterjee, Anticancer Res. 23 (2003) 3243–3246.
[51] S.L. Born, D. Caudill, B.J. Smith, L.D. Lehman-McKeeman, Toxicol. Sci. 58 (2000) 23–31.
[52] N.J.S. Fauzee, Z. Dong, Y. Wang, Asian Pacific J. Cancer Prev. 12 (2011) 837–851.
[53] L.M. Nunes, E. Robles-Escajeda, Y. Santiago-Vazquez, N.M. Ortega, C. Lema, A. Muro, G. Almodovar, U. Das, S. Das, J.R. Dimmock, R.J. Aguilera, A. Varela-Ramirez, AAPS J. 16 (2014) 872–874.
[54] J.K. Buolamwini, Curr. Pharm. Des. 6 (2000) 379–392.
[55] J.I. Chao, P.C. Kuo, T.S. Hsu, J. Biol. Chem. 279 (2004) 20267–20276.
[56] X. Xu, Y. Zhang, D. Qu, T. Jiang, S. Li, J. Exp. Clin. Cancer Res. 30 (2011) 33.
[57] J. Kahn, P. Preis, F. Waldman, A. Tseng Jr., J. Cancer Res. Clin. Oncol. 120 (Suppl) (1994) S19–S22.
[58] A. Rasul, M. Khan, B. Yu, T. Ma, H. Yang, Asian Pac. J. Cancer Prev. 12 (2011) 1219–1223.
[59] J.M. Millot, S. Sharonov, M. Manfait, Cytometry 17 (1994) 50–58.
[60] S. Jayaraman, J. Immunol. Methods 306 (2005) 68–79.
[61] E. Robles-Escajeda, D. Lerma, A.M. Nyakeriga, J.A. Ross, R.A. Kirken, R.J. Aguilera, A. Varela-Ramirez, PLoS ONE 8 (2013) e73508.