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# Discovering the structure–activity relationships of different O-prenylated coumarin derivatives as effective anticancer agents in human cervical cancer cells

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ABSTRACT

Cervical cancer remains one of the greatest life threatening diseases for women worldwide. Although chemotherapy is considered as a standard treatment for advanced cervical cancers, there are still some drawbacks in this procedure including side effects and acquired drug resistance, which necessitate further research on development of more effective agents with less side effects. Among natural compounds, coumarin derivatives have shown anticancer properties on various cancerous cells and coumarin ring has proven to have a paramount role in development of anticancer drugs.

Here, we aimed to establish the structure-activity relationships of eighteen O-prenylated coumarin derivatives and determined their anticancer properties on HeLa cervical cancer and HDF normal cells by MTT assay. Moreover, the mechanism of cell death induced by these compounds and their effects on cell cycle were studied using flow cytometry. MTT results indicated that twelve O-prenylated coumarin derivatives exhibited selective toxicity on HeLa cells, while they had no significant toxic effects on normal cells. Besides, flow cytometric analyses, showed that the selected compounds induced apoptosis in HeLa cells, and could also result to $G_1$ cell cycle arrest.

In conclusion, analyzing structural–activity relationships revealed that a prenylation substitution at position 6 of the coumarin ring greatly improved anticancer properties of these agents. As these derivatives exerted their cytotoxic effects *via* apoptosis and were not toxic on normal cells, they can be considered as effective anticancer agents for further preclinical experiments.

## 1. Introduction

Cancer is currently the second leading cause of death in the globe and its increasing incidence may even outrun cardiovascular diseases in future (Bray et al., 2018). Cancer is defined as a group of diseases characterized by uncontrolled growth of abnormal cells and their metastatic properties (Seyfried and Shelton, 2010). Cervical cancer is the fourth most common cancer in women (approximately 12%) and is generally the seventh most common cancer in the world. According to worldwide estimates, around half a million new cases are discovered annually with more incidence in developing countries (Mokdad et al., 2019). Socioeconomic differences and various causes such as low access to screening programs, not enough prevention strategies, ineffective and inadequate treatment regimens, and poor health conditions can be attributed to high geographical variations in the incidence and mortality of cervical cancer (Downs et al., 2008; Goel et al., 2003; Katz and Hofer, 1994). Although the incidence of this cancer is low in Iran and in many Muslim countries in comparison with other geographical areas, its mortality is high (Bray et al., 2018). This cancer is the 12th most common cancer among Iranian women, with around 947 new cases annually diagnosed. Forasmuch as the risk factors associated with this cancer are not few, cervical cancer requires special attention (Bruni et al., 2016).

In spite of many progress in treatment strategies including chemotherapy, radiotherapy and surgery, the mortality rate of cancer is still high (Miller et al., 2016; Siegel et al., 2016). Some problems associated with chemotherapy including acquired resistance or high rate of adverse effects on normal cells necessitate the search for new and

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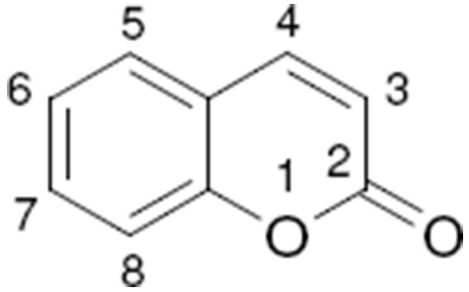



**Fig. 1.** Chemical structure of coumarin.

safer agents with anticancer properties.

Plant- derived compounds have played an important role in cancer chemotherapy for over 50 years. These compounds are often readily available and their safety and efficacy are reported in many studies (Butler et al., 2014; Cragg et al., 2011; Newman and Cragg, 2016). A problem associated with using herbal substitutes is their limited supply. However, naturally occurring scaffolds such as coumarins offer basic structures which can be further improved by synthesizing novel compounds to display a wide spectrum of pharmacological activities including anticancer, antibiotic, antidiabetic and others, by acting on multiple targets (Singh et al., 2019b).

Coumarins are a remarkable group of these natural products which consist of fused benzene and pyrone rings and comprise a very large class of phenolic derivatives (Fig. 1) (Kostova, 2007). Natural coumarins are known for their special pharmacological properties and synthetic coumarin analogs are also reported for antihypertensive (Bai et al., 2015), anticoagulant (Ghazaryan et al., 2007), Antibacterial (Bhagat et al., 2019; Singh et al., 2019a), antifungal (Sardari et al., 1999), antiviral (Shokoohinia et al., 2014), antihyperglycemic (Raju et al., 2010), anticancer (Haghighi et al., 2014; Singh et al., 2016; Singh et al., 2017; Haghighitalab et al., 2014), anti-inflammatory and analgesic properties (Peng et al., 2013; Wu et al., 2009)

The addition of a prenyl chain (C5 (isopentenyl), C10 (geranyl), C15 (farnesyl)) to a coumarin ring through oxygen atom often results in a derivative with improved biological and pharmacological properties such as antimicrobial, antitumor, and anti-inflammatory activities (Genovese et al., 2018). In the last three decades, 7-prenyloxycoumarins as a group of secondary metabolites found in families of Rutaceae and Umbelliferae with paramount and various biological and pharmacological activities, have gained the attention of researchers (Askari et al., 2009).

Auraptene (7-geranyloxycoumarin) is one of the most abundant O-prenylated coumarins occurring in nature. Different studies have demonstrated that auraptene possesses numerous pharmacological and medicinal properties including antidiabetic (Takahashi et al., 2011), antiprotozoal (Napolitano et al., 2004), anti-genotoxic (Soltani et al., 2010), immunomodulatory (Tanaka et al., 1999) and anti-inflammatory (Murakami et al., 2000) activities as well as significant effects on prevention and treatment of various chronic diseases (Derosa et al., 2016). It is also known for its chemopreventive (Tanaka et al., 1998) and antitumor properties in many types of cancers (Sakata et al., 2004; Murakami et al., 2000).

Umbelliprenin (7-farnesyloxycoumarin) with a structure close to auraptene is another naturally occurring O-prenylated coumarin. Previous researches have shown different promising biological and pharmacological activities of this compound including lipoxygenase inhibitory (Iranshahi et al., 2012), antioxidant (Patel and Patel, 2011), anti-inflammatory (Iranshahi et al., 2009a), chemopreventive (Iranshahi et al., 2009b), and cytotoxic properties (Barthomeuf et al., 2008).

Considering the pharmacological importance of synthetic coumarins and paramount biological and pharmacological activities of O-prenylated coumarins and the problems we are facing in cancer treatment in terms of lack of selectivity and specificity, the current study was aimed to evaluate *in vitro* antiproliferative effects of prenylated derivatives including isopentenyloxy, geranyloxy and farnesyloxy substituents at positions 3, 4, 5, 6, 7 and 8 of coumarin ring. Apoptotic inducing effects of these compounds were also evaluated on HeLa cells using FITC-annexin V and propidium iodide stainings and the structure–activity relationships (SAR) for these compounds were investigated. HDF (human dermal fibroblast), a non-cancerous cell line, was also used as a control to determine the anticancer properties of these O-prenylated coumarin derivatives.

## 2. Materials and methods

### 2.1. Synthesis of O-prenylated coumarin derivatives

Eighteen O-prenylated coumarin derivatives were synthesized as described previously (Iranshahi et al., 2012) (Table 1).

### 2.2. Preparing different solutions of O-prenylated coumarin derivatives

To prepare different concentrations of eighteen O-prenylated coumarin derivatives, 2 mg of each compound was dissolved in 100 µl dimethylsulfoxide (DMSO) (Merck, Germany) and diluted with complete culture medium before experiments. To have a better evaluation, the viability of each treatment was compared with control DMSO solution containing 0.25% DMSO in complete medium.

### 2.3. Culture of HeLa and HDF cells

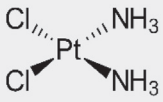
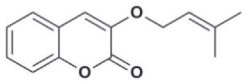
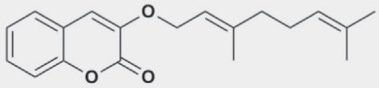
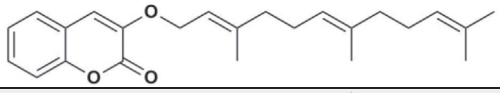
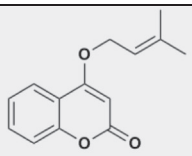
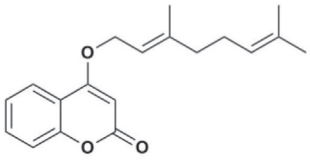
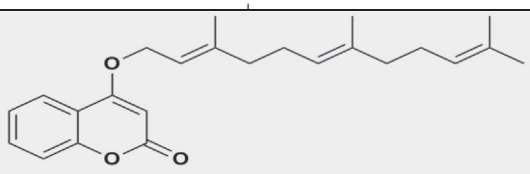
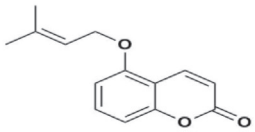
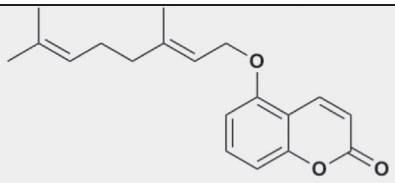
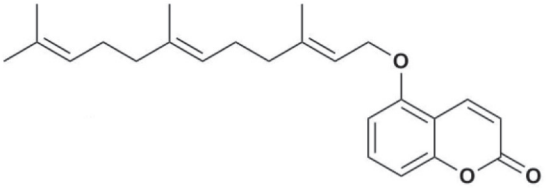
HeLa cells, obtained from Pasteur Institute (Tehran, Iran), were grown in Roswell Park Memorial Institute (RPMI 1640) medium (Thermo Fisher Scientific, Germany) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Germany). HDF cells, a generous gift from ACECR (Mashhad, Iran), were cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Germany) supplemented with 10% FBS. Both cell lines were maintained in a humidified atmosphere of 5% $CO_2$ at 37 °C and subcultured, when required, using 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Germany).

### 2.4. In vitro cytotoxicity assay

The *in vitro* tetrazolium-based colorimetric assay (MTT) is a rapid method based on the cleavage of a yellow tetrazolium salt (3-(4, 5-dimethylthiazol-2 yl)-2, 5 diphenyltetrazolium bromide) to purple formazan crystals by mitochondrial enzymes of metabolically active cells (Mosmann, 1983).

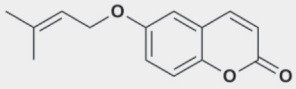
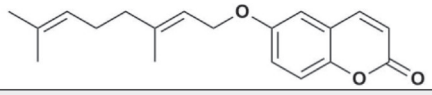
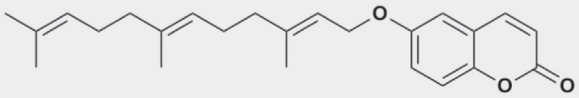
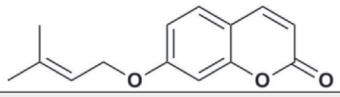
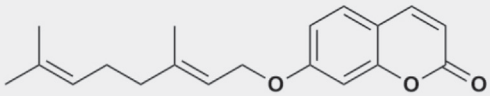
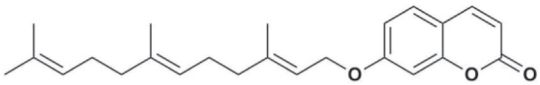
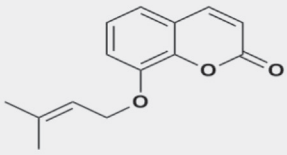
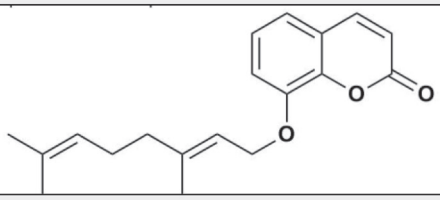
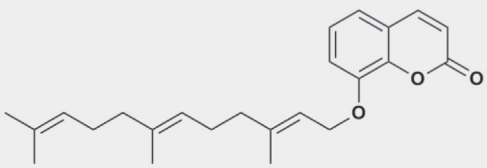
MTT assay was used to determine the half maximal inhibitory concentrations ($IC_{50}$) of O-prenylated coumarin derivatives as well as cisplatin on HeLa cells (for all compounds, the MTT assay was repeated at least three times). After that, the $IC_{50}$ values of the derivatives with significant cytotoxic effects on HeLa cells were determined on HDF cells. To do so, cells were seeded, at a density of 13,000 HeLa and 9000 HDF cells per well of 96-well tissue culture plates (Orange Scientific, France). After 24 h, both cell types were incubated with increasing concentrations of the compounds. Since the mentioned compounds were dissolved in DMSO, which is a cytotoxic solution itself, three wells containing 0.25% DMSO were considered as control in each experiment. In addition, cells were also treated with different concentrations (50, 25, 12.5, 6.25 and 3.125 µg/ml) of cisplatin as a positive control. After 24, 48 and 72 h treatments, MTT solution (5 mg/ml in PBS) (Sigma, Germany) was added to each well, and after 3 h of incubation, the resulting formazan was solubilized in 150 µl DMSO. The absorption was then measured at 540 nm in an ELISA reader (Awareness, USA). The percentages of living cells compared to the controls were calculated

**Table 1**
Chemical characteristics of eighteen O-prenylated coumarin derivatives and cisplatin.

| Compound | Chemical Structure | Molecular mass (g/mol) |
|---|---|---|
| Cisplatin |  | 300.01 |
| 3-Isopentenyloxycoumarin (3-IC) |  | 230 |
| 3-Geranyloxycoumarin (3-GC) |  | 298 |
| 3-Farnesyloxycoumarin (3-FC) |  | 366 |
| 4-Isopentenyloxycoumarin (4-IC) |  | 230 |
| 4- Geranyloxycoumarin (4-GC) |  | 298 |
| 4-Farnesyloxycoumarin (4-FC) |  | 366 |
| 5-Isopentenyloxycoumarin (5-IC) |  | 230 |
| 5-Geranyloxycoumarin (5-GC) |  | 298 |
| 5-Farnesyloxycoumarin (5-FC) |  | 366 |

(*continued on next page*)

**Table 1** (*continued*)

| Compound | Chemical Structure | Molecular mass (g/mol) |
|---|---|---|
| 6- Isopentenyloxycoumarin (6-IC) |  | 230 |
| 6-Geranyloxycoumarin (6-GC) |  | 298 |
| 6- Farnesyloxycoumarin (6-FC) |  | 366 |
| 7- Isopentenyloxycoumarin (7-IC) |  | 230 |
| 7-Geranyloxycoumarin (7-GC) Auraptene |  | 298 |
| 7- Farnesyloxycoumarin (7-FC) Umbelliprenin |  | 366 |
| 8- Isopentenyloxycoumarin (8-IC) |  | 230 |
| 8-Geranyloxycoumarin (8-GC) |  | 298 |
| 8- Farnesyloxycoumarin (8-FC) |  | 366 |

according to the following equation for different treatments:

%viability of cells
= the mean absorbance of treated cells in each well /the mean absorbance of control cells (DMSO) × 100.

### *2.5. Investigating apoptosis inducing effects by flow cytometry*

Apoptosis was assessed in HeLa cells using fluorescein isothiocyanate (FITC) annexin V apoptosis detection kit with propidium iodide (BioLegend, USA) according to manufacturer's instructions. Briefly, following each treatment, cells were collected, washed, and resuspended in a staining buffer. Then, samples were stained with FITC-

Annexin V and propidium iodide for 15 min at room temperature in dark, followed by addition of binding buffer. Finally, cells were analyzed by a flow cytometer (BD Accuri C6, USA) using FL1 and FL2 filters. Considering the importance of studying the early stages of apoptosis, the 48 h time interval was selected as the suitable time for sampling. Moreover, the mechanism of cisplatin-induced cell death was also studied for comparison. Furthermore, control samples including untreated and unstained HeLa cells as well as cells treated with DMSO were employed to eliminate the diffusion effects of dyes bound non-selectively to cells and also to eliminate the effects of DMSO solvent. Results of flow cytometry were analyzed using FlowJo 7.6.1 software.

### 2.6. Cell cycle analysis

For cell cycle analysis, HeLa cells were treated with $IC_{50}$ concentrations of selected O-prenylated coumarin derivatives for 48 h. Cells were then trypsinized and washed with cold phosphate buffered saline (PBS) and fixed with 70% ethanol. A solution including 350 µl of cold PBS, 50 µl triton X-100 (1%), 20 µl RNase (0.2 mg/ml) (Sigma, Germany) and 30 µl propidium iodide (100 mg/ml) (Sigma, Germany) was added to the cell suspensions and cells were kept on ice for 15 min in a dark place. Then the samples were analyzed with a flow cytometer (BD biosciences, San Jose, CA, USA). The percentages of cells in $G_1$, S and $G_2$ phases of cell cycle were determined and analyzed using FlowJo 7.6.1 software.

## 3. Results

### 3.1. Significant cytotoxic effects of O-prenylated coumarin derivatives on HeLa cells

In present study cytotoxic and anticancer properties of O-prenylated coumarin derivatives (Table 1) were investigated on HeLa cervical cancer cells by MTT assay. Considering the length of the carbon chain, these compounds were divided into three types: C5 (isopentenyloxy analogs), C10 (geranyloxy analogs) and C15 (farnesyloxy analogs) groups.

The calculated $IC_{50}$ values for different time points are shown in Table 2 for the three mentioned groups. To compare cytotoxic effects of O-prenylated coumarin derivatives with those of the commonly used drugs in the clinic, cisplatin (a general and well-known cytotoxic compound) was employed as a positive control (Table 2 and Fig. 2).

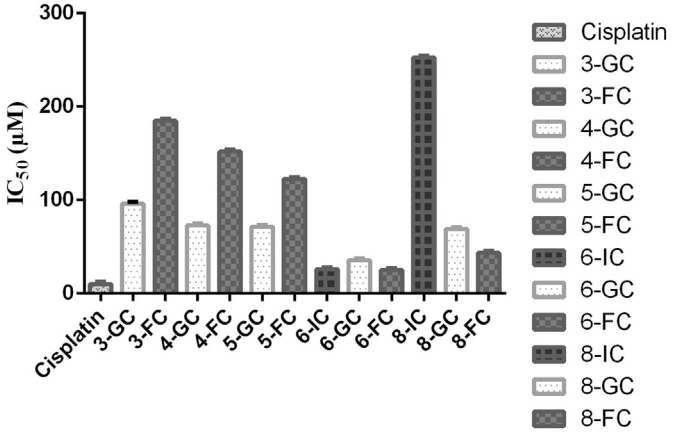



**Fig. 2.** Comparison between anti-proliferative activity of cisplatin and selected O-prenylated coumarin derivatives on HeLa cells, as determined by MTT assay after 72 h. $IC_{50}$ values are shown as mean ± SD (n = 3).

After exposing HeLa cells to various concentrations of cisplatin, the $IC_{50}$ values of 11.05, 5.806, and 3.928 µg/ml equivalent to 35, 17, and 10 µM were obtained at the time intervals of 24, 48 and 72 h, respectively.

### 3.2. Selected O-prenylated coumarin derivatives had no significant toxic effects on normal HDF cells

O-prenylated coumarin derivatives which exhibited significant effects on HeLa cells were used to determine their $IC_{50}$ values on HDF cells (Table 2). The results of MTT assay indicated that these compounds had no cytotoxic effects on normal HDF cells. As shown in Table 2, the $IC_{50}$ values of cisplatin on HDF cells were determined as 65.35 µg/ml (218 µM), 48.07 µg/ml (160 µM) and 32.445 µg/ml (108 µM) after 24, 48 and 72 h of treatments, respectively.

### 3.3. Induction of apoptosis in HeLa cells by O-prenylated coumarin derivatives

FITC-Annexin V and PI staining assay was used to study the mechanism of cell death induced by the studied compounds, and the stained cells were analyzed by a flow cytometer with FL1 filter for FITC

**Table 2**
Anti-proliferative activity of O-prenylated coumarin derivatives on HeLa and HDF cells, as determined by MTT assay. $IC_{50}$ values are shown as mean ± SD ($n$ = 3). ND = Not Determined.

| Compound | Concentration range | $IC_{50}$ (µM) ± SD (HeLa) | | | $IC_{50}$ (µM) ± SD (HDF) | | |
|---|---|---|---|---|---|---|---|
| | | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| Cisplatin | 3.125–50 µg/ml | 35 ± 1.92 | 17 ± 2.05 | 10 ± 2.40 | 218 ± 2.19 | 160 ± 1.96 | 108 ± 2.09 |
| (3-IC) | 1.562–50 µg/ml | – | – | – | ND | ND | ND |
| (3-GC) | 1.562–50 µg/ml | 433.7 ± 2.21 | 218.8 ± 1.97 | 96 ± 2.04 | – | – | – |
| (3-FC) | 1.562–50 µg/ml | – | 359.8 ± 2.78 | 184.8 ± 2.00 | – | – | – |
| (4-IC) | 1.562–50 µg/ml | – | – | – | ND | ND | ND |
| (4-GC) | 1.562–50 µg/ml | 627.2 ± 1.81 | 163.9 ± 1.88 | 72.79 ± 1.98 | – | – | – |
| (4-FC) | 1.562–50 µg/ml | 462.2 ± 2.61 | 200 ± 1.90 | 151.9 ± 1.86 | – | – | – |
| (5-IC) | 1.562–50 µg/ml | – | – | – | ND | ND | ND |
| (5-GC) | 1.562–25 µg/ml | 152.2 ± 1.88 | 86.46 ± 2.16 | 71.70 ± 1.76 | – | – | – |
| (5-FC) | 1.562–50 µg/ml | – | 139.7 ± 1.85 | 122.7 ± 1.95 | – | – | – |
| (6-IC) | 1.562–12.5 µg/ml | 94.83 ± 2.12 | 33.44 ± 1.74 | 26.19 ± 1.84 | – | – | – |
| (6-GC) | 1.562–50 µg/ml | 172.2 ± 1.8 | 47.10 ± 1.97 | 35.74 ± 1.88 | – | – | – |
| (6-FC) | 1.562–25 µg/ml | 143 ± 2.19 | 38.30 ± 2.11 | 25.24 ± 1.90 | – | – | – |
| (7-IC) | 1.562–50 µg/ml | – | – | – | ND | ND | ND |
| (7-GC) | 6.5–100 µg/ml | 699.9 ± 2.14 | 650 ± 2.46 | 556.6 ± 2.13 | ND | ND | ND |
| (7-FC) | 6.25–50 µg/ml | 579.5 ± 2.73 | 516.66 ± 2.66 | 251.4 ± 2.47 | ND | ND | ND |
| (8-IC) | 1.562–50 µg/ml | – | 258.5 ± 1.89 | 252.6 ± 2.08 | – | – | – |
| (8-GC) | 1.562–25 µg/ml | 136.4 ± 1.90 | 74.82 ± 1.76 | 69.21 ± 1.77 | – | – | – |
| (8-FC) | 1.562–50 µg/ml | – | 82.38 ± 1.90 | 43.48 ± 1.92 | – | – | – |

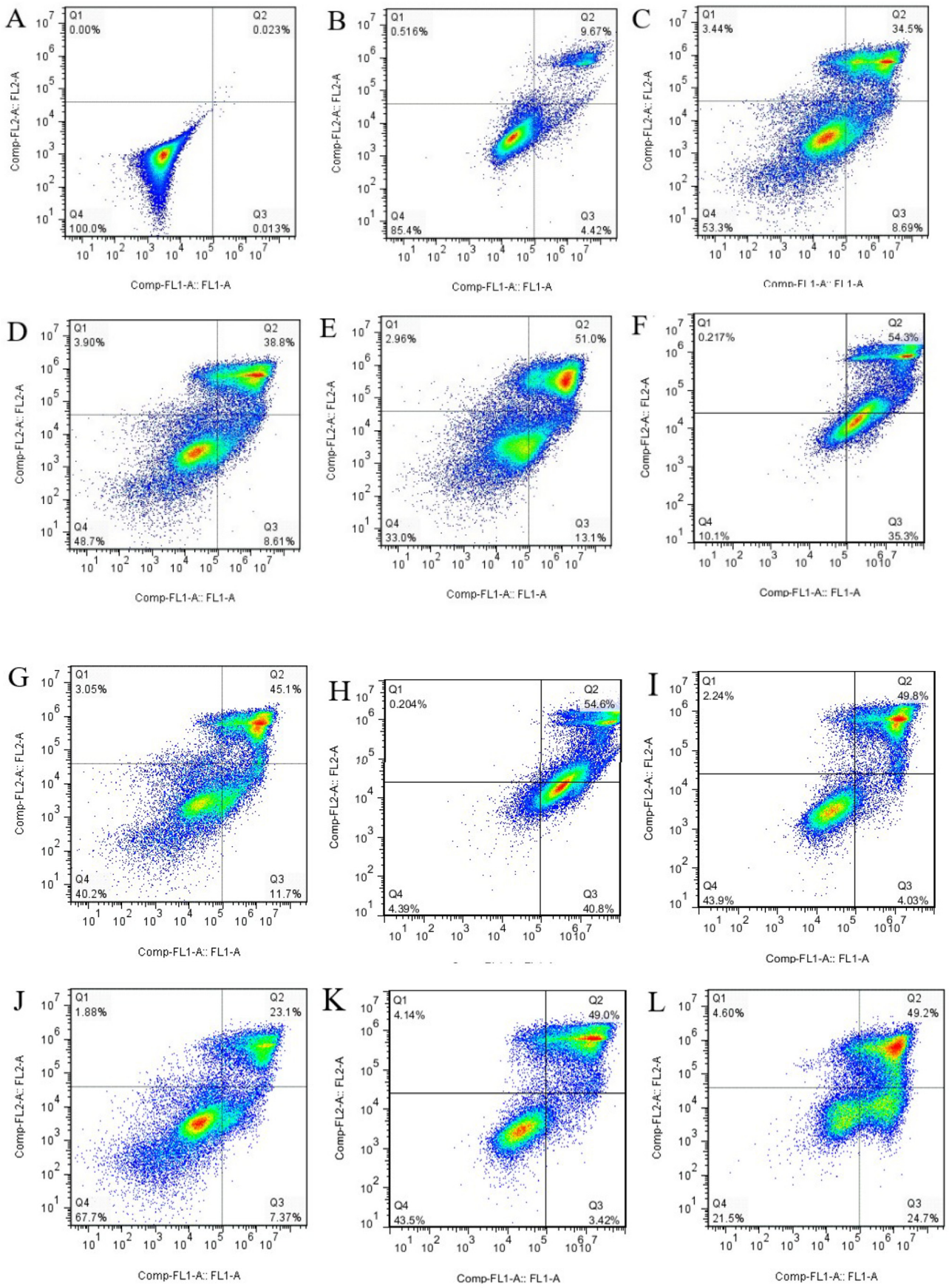



**Fig. 3.** Selected O-prenylated coumarin derivatives could induce apoptosis in HeLa cervical cancer cells. Apoptotic cells were detected with FITC-Annexin V and PI stainings at 48 h after treatets A: Untreated, B: DMSO, C: 3-GC, D: 4-GC, E: 5-GC, F: 6-IC, G: 6-GC, H: 6-FC, I: 8-IC, J: 8-GC, K: 8-FC, L: Cisplatin.

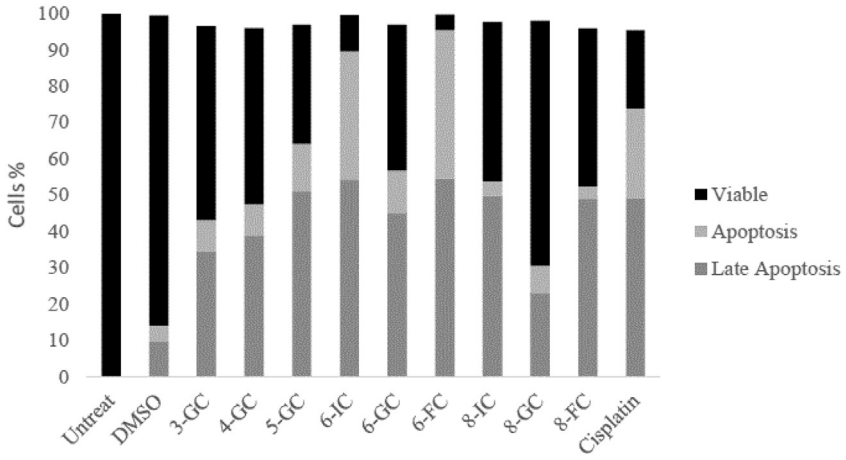



**Fig. 4.** Comparison of apoptosis induced by selected O-prenylated coumarin derivatives. Data are expressed as percentage of cells placed in each quadrant.

and FL2 filter for PI. Results are presented in Figs. 3 and 4.

### 3.4. Selected O-prenylated coumarins induced cell cycle arrest at $G_1$ phase

The status of the cell cycle of HeLa cells treated with selected O-prenylated coumarins (6-IC: 33.44 μM; 48 h, 6-GC: 47.10 μM; 48 h and 6-FC: 38.30 μM; 48 h) were analyzed by PI staining and flow cytometry. As shown in Fig. 5, selected O-prenylated coumarins induced cell cycle arrest at $G_1$ phase along with a decrease in S phase.

## 4. Discussion

Since, available standard chemotherapy regimens do not offer adequate survival benefits, it has become necessary to develop new therapeutic agents to improve treatment efficacy of various cancers. For many chemotherapeutic agents, a direct correlation between the anti-tumor effectiveness and the ability to induce apoptosis has been established, and the development of new anticancer approaches, aimed to promote apoptosis in cancer cells, has gained a paramount importance.

In medicinal chemistry, natural products, with the ability to interact with more than one target, represent a significant source of inspiration for designing structural analogs with improved pharmacological profiles (Belluti et al., 2010).

The coumarin class of organic compounds consists of a 1, 2-benzopyrone ring as a basic parent scaffold (Venkata Sairam et al., 2016). Coumarins are characterized by low molecular weight, easy synthesis and high bioavailability, as well as a variety of pharmacological activities (Haghighitalab et al., 2014; Peng et al., 2013; Wu et al., 2009). Investigating the relationship between chemical structure and pharmacological effects of coumarin compounds can be the basis for designing novel and more effective drugs.

Despite advances in early screening and prevention of cervical cancer, prognosis of this malignant tumor is particularly poor and 1-year survival rate of advanced/recurrent patients is only 10–20% (Diaz-Padilla et al., 2013). The occurrence of drug resistance is the main cause of treatment failure and its related deaths. Therefore, development of more effective agents is required to overcome this life-threatening disease.

Considering promising pharmacological properties of coumarins and the importance of studying structure-activity relationships, in this study eighteen O-prenylated coumarin derivatives were synthesized and their cytotoxic and anticancer properties were investigated on HeLa cervical cancer and HDF normal cells by MTT assay. Moreover, the mechanism of cell death induced by these compounds was studied using FITC-Annexin V and PI stainings.

### 4.1. O-prenylated coumarin derivatives had selective cytotoxic effects on HeLa cells

As shown in Table 2 some derivatives had significant dose dependent cytotoxic effects on HeLa cells. However, it was interesting to see that selected O-prenylated coumarin derivatives with significant

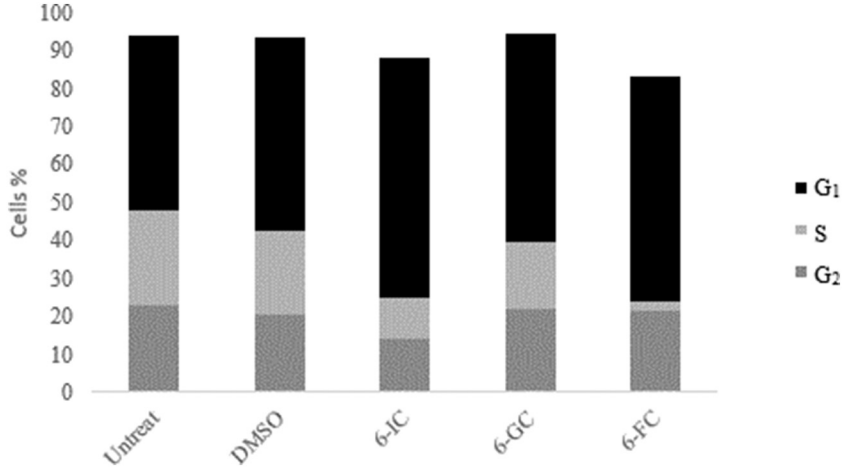



**Fig. 5.** 6-IC, 6-GC and 6-FC induced cell cycle arrest at $G_1$ phase, as shown by PI staining. HeLa cells treated with 0.25% DMSO, 6-IC (33.44 μM), 6-GC (47.10 μM) and 6-FC (38.30 μM) for 48 h were subjected to PI staining and flow cytometry.

cytotoxic effects on Hela cells, had no inhibitory effects on the growth of HDF normal cells. In total, twelve O-prenylated coumarin derivatives exhibited selective toxicity on HeLa cancerous cells. The cytotoxic effects of some of these compounds have been investigated on different cancerous cells previously. Orafaei et al. reported that $IC_{50}$ values of 5-farnesyloxycoumarin (5-FC) on PC-3 cells were 40.1, 27.05, and 26.43 μg/ml, while they were 124.02, 101.7, and 81.42 μg/ml on normal fibroblast cells after 24, 48 and 72 h, respectively (Orafaie et al., 2016). 6-farnesyloxycoumarin, 7-farnesyloxycoumarin and 8-farnesyloxycoumarin have shown similar cytotoxic properties in prostate cancer cells (Hosseinymehr et al., 2016; Saboormaleki et al., 2018). Results obtained from the mentioned studies demonstrated that O-prenylated coumarin derivatives could have selective toxicity on prostate cancer cells.

### 4.2. Anti-cancer properties of O-prenylated coumarin derivatives are induced via apoptosis

Use of Annexin V and PI stainings is a quick method for detection of early apoptosis (Sgonc and Gruber, 1998). The redistribution of phosphatidylserine (PS) to the external side of the cell membrane occurs due to perturbation in the cell membrane. Annexin V, a recombinant phosphatidylserine binding protein, interacts mainly with PS residues, and it is used in conjunction with fluorochromes for detecting apoptosis using flow cytometry (Arur et al., 2003; Huerta et al., 2007). In the present study, it was found that selected O-prenylated coumarin derivatives inhibited HeLa cells by inducing apoptosis. The percentage of apoptosis quantified by flow cytometry analysis showed that, the vast majority of HeLa cells in the untreated and DMSO controls remained unstained, while cells treated with selected compounds were significantly more positive for Annexin V, indicating apoptosis as the main mechanism of cell death (Fig. 3).

### 4.3. Selected O-prenylated coumarins result to $G_1$ arrest

The effects of 6-IC, 6-GC and 6-FC on the cell cycle of HeLa cells were also analyzed by flow cytometry. As shown in Fig. 5, exposure of HeLa cells to mentioned compounds caused cell cycle arrest at $G_1$ phase along with a decrease in S phase. Different studies have demonstrated considerable evidences that most of investigated coumarins could induce cell cycle arrest at $G_1$ phase. It was shown that 7,8-diacetoxy-4-methylcoumarin (DAMC) and one of its derivatives, induced $G_1$ arrest in A549 cells (lung cancer) *via* inhibition of MAPK signaling pathway and activation of NF-κB (Goel et al., 2009). Esculetin (100 μM) also induced $G_1$ arrest in HL-60 cell line (human leukemia) (Wang et al., 2002). Furthermore, umbelliprenin (*7*-farnesyloxycoumarin) also arrested M4Beu and PC-3 cells at $G_1$ phase (Barthomeuf et al., 2008; Saboormaleki et al., 2018). It was also reported that 5-farnesyloxycoumarin and 8-farnesyloxycoumarin induced cell cycle arrest at $G_1$ phase in PC-3 cells (Orafaie et al., 2017; Hosseinymehr et al., 2016). Besides, Chuang et al. studied the effects of coumarin on cell viability, cell cycle arrest and induction of apoptosis in HeLa cells. Their results indicated that coumarin treatment gradually decreased the expression of $G_1$-associated proteins which may have led to the arrest in these cells (Chuang et al., 2007).

### 4.4. Investigating the relationship between structure and cytotoxic effects of O-prenylated coumarin derivatives on HeLa cells

Coumarin and its metabolic derivatives comprise a large class of phenolic compounds in plants, and they exhibit extensive pharmacological activities. Therefore, several studies have used coumarin as a lead compound to design novel structure based drugs. In this study, eighteen O-prenylated coumarin derivatives were used to analyze their structure–activity relationship (SAR). As shown in Table 1, these compounds were compared in terms of positions and lengths of the prenyl chains attached to the main coumarin skeleton.

Our results demonstrated that adding the side-chain functional groups to the C-6 position could result to compounds with more anticancer properties. This presents a new platform for synthesizing novel substances with anticancer properties. Moreover, our results indicated that C-8 can also be introduced as an important position on the coumarin backbone after C-6 position. Different studies have introduced C-6 as an important position for various bioactivities of coumarins (Zhu and Jiang, 2018).

Prenylation is a chemical or biochemical reaction in which prenyl groups are added to a molecule. In particular, prenylation of aromatic molecules plays a critical role in the biosynthesis of a wide range of secondary metabolites exerting valuable pharmacological effects across phylogenetically different classes of living organisms, from bacteria to plants and mammals.

O-prenylated natural products belong to a family of secondary metabolites that have been considered for years just as biosynthetic intermediates of C-prenylated derivatives. A wide variety of compounds containing a prenyloxy side chain have been isolated including coumarins. Considering the length of the carbon chain, these compounds were divided into three types: C5 (isopentenyloxy analogs), C10 (geranyloxy analogs) and C15 (farnesyloxy analogs). Many of the isolated oxyprenylated natural products and their semisynthetic derivatives are shown to exert remarkable anticancer, anti-inflammatory, antimicrobial and antifungal effects both *in vitro* and *in vivo*. For instance, Mousavi et al. showed that the presence of a hydrophobic chain at the C7-OH position of the 1, 2-benzopyrone ring played an important role in cytotoxicity of aurapten and umbelliprenin on MCF-7, a breast carcinoma cell line. This chain increased the lipophilic property and thus facilitated their infiltration into cells. The absence of this group in the structure of umbelliferone reduced its cytotoxicity. Furthermore, herniarin (*O*-methyl umbelliferone) with methyl substitution at 7-OH has more hydrophobic groups than umbelliferone and was more toxic to the studied cells (Fig. 6) (Mousavi et al., 2015).

Thus, it can be concluded that cytotoxicity exhibited by these compounds will also differ based on the lengths of the prenyl chains. Except for the 6P and 8P compounds, isopentenyloxycoumarins did not show considerable toxic effects on HeLa cells because of their short prenyl groups. The substantial toxicity of these two substances can be attributed to the fact that their prenyl groups are situated at the two well-known pharmacophor C-6 and C-8 positions, and we consider this toxicity level solely resulting from the important attachment position and not from prenyl moiety length. Moreover, results indicated that farnesyloxycoumarins had considerable anticancer effects, especially when situated at the 6 and 8 positions of the coumarin. However, it must be noted that the long carbon chains in these compounds led to less cytotoxicity on HeLa cells during the first 24 h probably due to reducing their penetration rate into cells.

Studies on geranyloxycoumarins showed that apart from 6-GC and

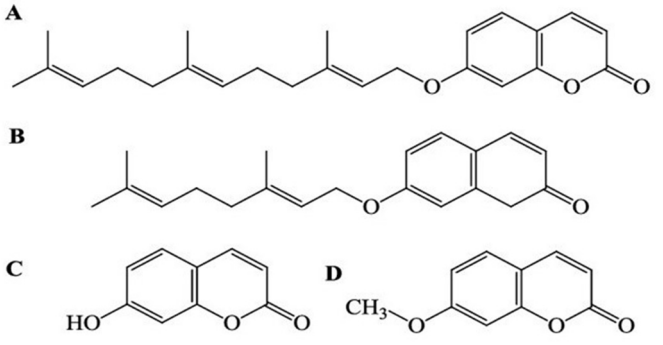



**Fig. 6.** Chemical structure of prenyloxy coumarin compounds. A: Umbelliprenin, B: Auraptene, C: Umbelliferone and D: Herniarin.

8-GC which had the highest toxicity in this group, other positions also had significant toxicity on cancerous cells that could be attributed to the suitable length of the geranyl chain. Further studies on the effects of these compounds on other cancerous cells and also in animal models can better elucidate their structure- activity relationships.

## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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