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Original Article

Cytotoxic Effects of Hydroxy Coumarin Derivatives on Mouse Neuroblastoma N2a Cell Line

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Abstract

Neuroblastoma is one of the nervous system cancers, which approximately consists of 9% of childhood cancers. In this study, we evaluated the toxic effects of prenyl hydroxy coumarin derivatives on apoptosis of the neuroblastoma cell line N2A. N2A cells were cultured in DMEM medium, then the effects of different concentrations (0.75–200 µg/mL) of prenyl hydroxy coumarin derivatives during 24, 48, and 72 h were studied. Cell viability was quantified by MTT assay; apoptotic cells were determined using PI staining of DNA fragmentation through flow cytometry (sub-G1 peak). The toxic effect of 3- farnesyl oxi coumarin in the N2A cell starts at 6.25 µg/ml and increases relatively depending on rising in concentration and over time. The toxicity and apoptosis of 3- farnesyl and 6- farnesyl oxi coumarin is more than 3- Geranyl and 6-Geranyl oxi coumarin. Prenyl hydroxy coumarin induces peak sub-G1 in flow cytometry compared to the control group, indicating that induced toxicity, which is involved in apoptotic cell death. Different concentrations of derivatives (0.75-200 µg/mL) in lymphocytes did not induce any anti-proliferative effect in 24 h. In conclusion, prenyl hydroxy coumarin derivatives induce apoptotic effects in the N2A cell line. Then prenyl hydroxy coumarin derivatives sound to be chemotherapeutic agents for the neuroblastoma cancer cells.

Keywords: Apoptosis, N2A cell line, Prenyl hydroxy coumarin derivatives.

1. Introduction

Cancer is currently one of the leading causes of death in the world, with around 14 million new cases in 2012 and approximately 9.6 million deaths worldwide [1]. There will be 21.7 million new cancer cases and about 13.1 million cancer deaths in 2030 [2]. Anticancer drugs prevent the uncontrolled division of cancer cells, through DNA intercalating, cross-linking of DNA cancer

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cells, inhibition of DNA topoisomerase, and disruptors in the formation of cytoskeleton cancer cells [3]. Breast cancer is one of the major and deadly cancers, which is the second most common cancer in women and also the fourth most common cause of mortality and morbidity [4]. NF-kB is a transcription factor that inhibits the activation of apoptosis, which is inhibited by (S)-ginsenoside Rg3 (SRg3) in the MDA-MB-231 cell line [5]. Another study on the combination of Bacopaside (bac) I and II on the MDA-MB-231 class induced apoptosis and inhibited proliferation [6].

Neuroblastoma is one of the most common cancers in children. It is a neuroendocrine tumor, often originating from the sympathetic nervous system (SNS) [7]. About 50% of these tumors appear in the adrenal medulla, and the remaining 50% are in the paraspinal sympathetic ganglia region of the neck, chest, abdomen, and pelvis [8]. Neuroblastoma is one of the most common types of cancers in childhood, accounting for about 7-10 percent of all childhood cancers, and averaging about one per 7,000 live births each year, so about 700 new cases are reported in the United States each year [8-10].

The prevalence of neuroblastoma is uniform throughout the world (at least for western societies); the median age of diagnosis is 18 months [8, 11]. The etiology of this disease is still unknown, but about 40% at 12 months old and about 75% and 98% are diagnosed at 4 and 10 years, respectively, which are probably not affected by the environment [11].

Apoptosis is a physiological cell death that is essential for the evolution and homeostasis of the tissues [12]. Therefore, apoptotic pathways can be therapeutically exploited for cancer treatment and regulated by the balance between pro- and anti-apoptotic ones [13]. Some anticancer drugs induce apoptotic pathways in cancer cell [14]. Anticancer drugs have many characteristics that include: first, having high efficacy at usage in targeted therapies (drugs that specifically inhibit the growth of cancer cells); second, they have low toxicity for body organs [15]. Antioxidants for a long time have been effective agents in the prevention and medication of many diseases, including cancer. Therefore, many fruits and vegetables are sources of abundant antioxidants [16, 17]. Coumarin is a phenolic compound, which has a double-ring that consists of benzene and α-pyrones [18]. It naturally contains the extract of cassia leaf oil, lavender oil, Rutaceae, and Umbelliferae [18, 19]. Interestingly, coumarin also exists in microorganisms, such as Streptomyces and Aspergillus species [20, 21].

Coumarin has extensive effects on the body tissues, including different biological effects: anti-inflammatory, antimicrobial,

antioxidant, anti-tumor, anti–influenza, and antihyperlipidemic activity [22-27].

In this study, we evaluate the anti-proliferative effects of complete series of prenyl hydroxy coumarin derivatives such as 3-farnesyl oxi coumarin, 3-geranyl oxi coumarin, 6-farnesyl oxi coumarin, and 6-geranyl oxi coumarin on the mouse neuroblastoma N2A cell line and human lymphocytes [28].

2. Materials and Methods

2.1. Chemicals

Sodium citrate, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT), Trypan blue, Triton X-100, and the fluorescent probe propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM and FCS were purchased from Gibco (Grand Island, NY, USA).

2.2. Cell culture and Experiments

All experiments in this study were performed on mice neuroblastoma N2A cell lines (tumor-like neuroblasts). This cell lines were provided by the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran, Tehran) and cultured in Dulbecco Modified Eagle's Medium (DMEM). The N2A cells were cultured in DMEM media with FBS 10% and Penicillin-Streptomycin solution (100 unit/ml). The cells were maintained to 95% air and 5% CO_2 at 37°C in a humidified incubator. The cells were passaged every 3-4 days with trypsin EDTA in a flask.

Human lymphocytes were isolated from heparinized blood of healthy volunteers by Ficoll-Hypaque density gradient centrifugation. They were suspended at a density of 1.0 $\times 10^6$ cells/ml in a complete DMEM medium and cultured, as described previously[29].

2.3. Cell Viability

The MTT (*3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium* Bromide) assay is used to determine cell viability[30]. In brief, 10000 cell/well were seeded into flat-bottom 96-well cell culture plates and were incubated overnight into 95% air and 5%CO_2 at 37°C in a humidified incubator. Then cells were treated with different concentrations (0.75–200 µg/mL) of 3-Farnesyl oxi coumarin, 3-Geranyl oxi coumarin, 6-Farnesyl oxi coumarin and 6-Geranyl oxi coumarin for 24, 48 and 72 hours. After incubation, the supernatant was removed and, MTT (5 mg/ml in PBS) was added (the plate was wrapped with aluminum foil to prevent light exposure) then it was kept in the incubator for 4 hours. In the final step, 100 µl of DMSO was added and incubated for 3-5 minutes with gentle shaking (we made sure the crystals entirely dissolved). The absorbance was measured at 570 nm by ELISA reader (Stat fox).

2.4. Staining DNA Using Propidium Iodide

Apoptotic cells were detected using PI staining of cells by flow cytometry to detect the so-called sub-G1 peak[31]. Briefly, N2A cells were seeded 10^5 cell/well into a 24-well plate and allowed them to grow overnight. They were treated with different concentrations of (0.75–200 µg/mL) 3-farnesyl

oxi coumarin, 3-geranyl oxi coumarin, 6-farnesyl oxi coumarin and 6-geranyl oxi coumarin for 48 hours. The floating and adherent cells were harvested and incubated at 4°C overnight in the dark place using 750 µL of a hypotonic buffer containing (50 µg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100). The number of cells in the staining solution should be at a constant ratio of 1×10^6 cells per ml of hypotonic PI staining solution. Then they should be analyzed by a BD FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA, USA). About 10,000 events were acquired with FACS [32].

2.5. Statistical Analysis

All data are presented as Mean ± SEM from 3 experiments. One-way analysis of variance (ANOVA), followed by the Bonferroni post-test for multiple-group comparisons were used for data analysis. A p-value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Effects of Hydroxy Coumarin Derivatives on Cell Proliferation and Viability of Tumor Cells

Prenyl hydroxy coumarin derivatives including 3-Farnesyl oxi coumarin, 3-Geranyl

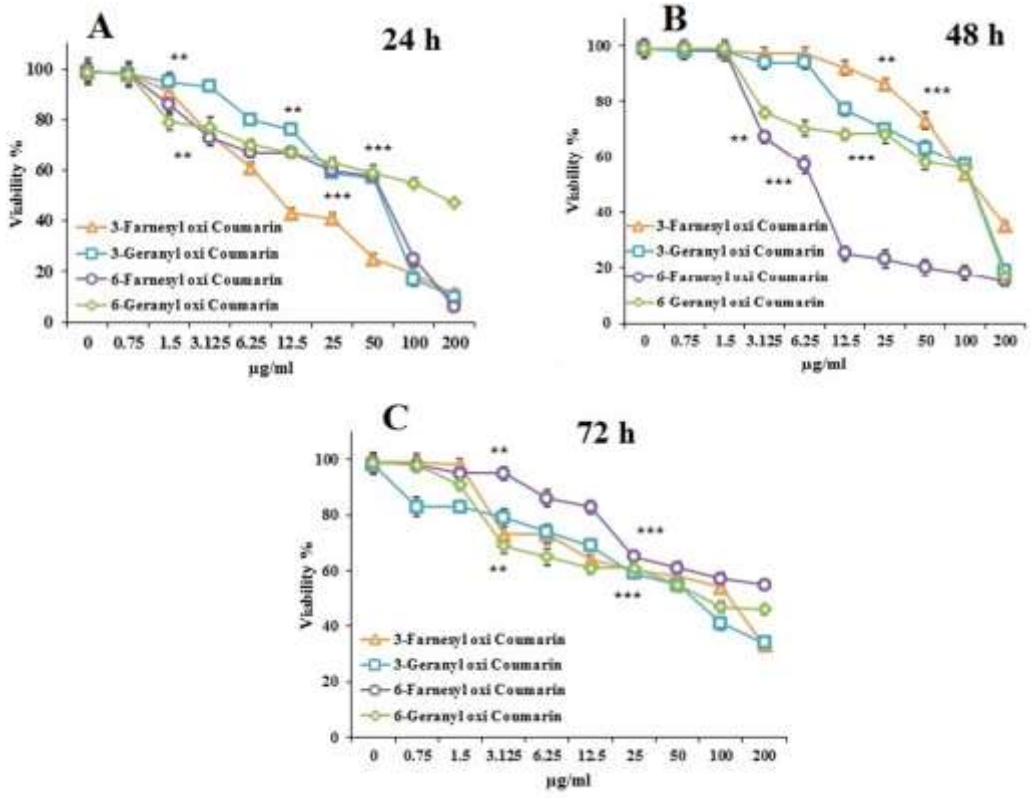

Figure 1. Cell survival rate of N2A cells evaluated by MTT. The cells at a density of 10^6cells/mL were cultured for 24 h, 48h and 72h in the absence and presence of different concentrations of Prenyl hydroxy comarnine derivatives (3-Farnesyl oxi coumarin , 3-Geranyl oxi coumarin, 6-Farnesyl oxi coumarin and 6-Geranyl oxi coumarin) (0–200 µg/mL). Data are presented as means ± SD of three independent experiments. Profiles A ,B and C are 24h , 48 and 72h , respectively. ∗p <0.05, ∗∗p <0.01, and ∗∗∗p <0.001 compared to control.

oxi coumarin, 6-Farnesyl oxi coumarin, and 6-Geranyl oxi coumarin (0.75–200 μg/mL) have dose-dependent cytotoxic effects on neuroblastoma cells (Figure 1). Therefore, the coumarin derivatives have enhanced concentration-dependent cytotoxic effects that the farnesyl hydroxy coumarin derivatives (3-Farnesyl oxi coumarin and 6-Farnesyl oxi coumarin) and the geranyl hydroxy coumarin derivatives (3-Geranyl oxi coumarin and 6-Geranyl oxi coumarin) exhibit the most anti-proliferative properties in 24 hours and 72 hours, respectively (Figure 1: A and C). Also, there is a significant difference between the neuroblastoma cells treated with Prenyl hydroxy coumarin derivatives (The IC_{50} values of these derivatives against N2A cell lines after 24 h,48h and 72h were determined (shown in Table 1, after 24, 48 and 72 hours ($P < 0.001$)).

cells when compared to the control, thus suggesting that apoptotic cell death is involved in its toxicity. IC_{50} values which were obtained from flow cytometry histogram of 6-Farnesyl oxi coumarin, 6- Geranyl oxi coumarin, 3-Farnesyl oxi coumarin and 3-Geranyl oxi coumarin in 48h were 35.8 μg/ml, 47.5 μg/ml, 98.4 μg/ml, and 197.2 μg/ml, respectively. Thus toxicity and apoptosis in compounds such as 6- Farnesyl oxi coumarin and 6-Geranyl oxi coumarin are more than 3-Farnesyl oxi coumarin and 3-Geranyl oxi coumarin. Results indicate a prenyl hydroxy comarnin derivative induced apoptosis in a concentration-dependent manner (Figure 2). As shown in Fig. 2, the Prenyl hydroxy coumarin derivative induces and also loses the membrane integrity of the cell at various concentrations of these Prenyl hydroxy comarnin derivatives in 48 hours.

Table 1. Doses inducing IC50 of Prenyl hydroxy comarnine derivatives against N2A cell lines.

Cell line	Compound/drugs	24h	48h	72h
N2A	3-Farnesyl oxi coumarin (μg/ml)	12.6±2.1	82.8±1.9	188.4±2.6
	6-Farensyl oxi coumarin (μg/ml)	9.25±1.8	37.5±1.2	83.6±1.9
	3-Geranyl oxi coumarin (μg/ml)	83.2±3.2	132.5±1.6	128.2±2.2
	6-Geranyl oxi coumarin (μg/ml)	68.9±1.8	25±1.1	95.6±1.8

The cells incubated with different concentration of Prenyl hydroxy comarnine derivatives for 24 h, 48h and 72h. IC50 values were expressed as the mean ± SD (n = 3)

3.2. Effects of Prenyl Hydroxy Coumarin Derivatives on Apoptosis in Neuroblastoma Cells

Prenyl hydroxy coumarn derivatives (IC_{50}, low, and more concentration) induce a sub-G1 peak in the flowcytometry histogram of treated

3.3. Effects of Hydroxy Coumarin Derivatives in Normal Cells

Natural coumarin and coumarin derivatives as anticancer agents have been investigated by many researchers [33-37]. Coumarins have excellent properties, such as the ease of

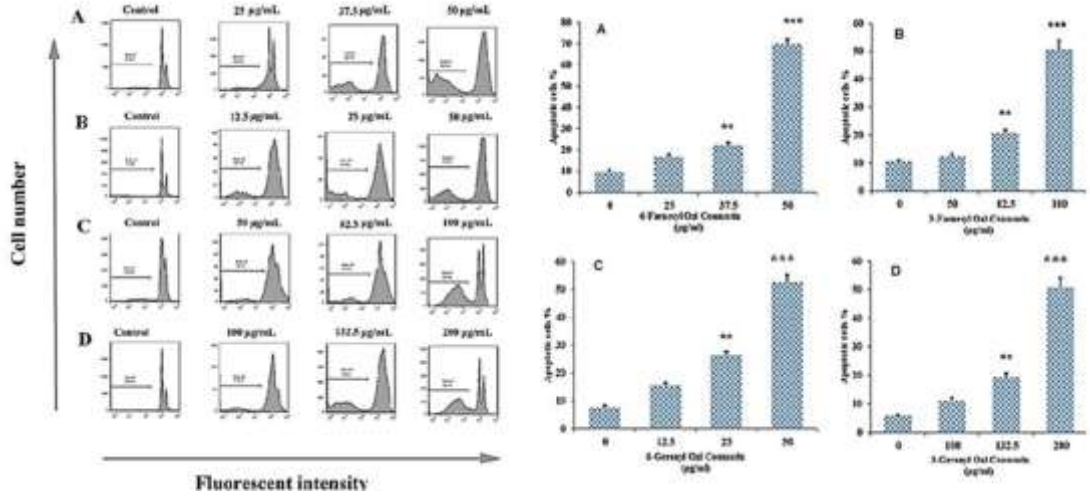

Figure 2. Flow cytometry profiles of the cells 48 h after Prenyl hydroxy comarnine derivatives exposure, stained with PI. Data are means ± SD of three experiments. Cells were treated with IC_{50}, low and more concentration of Prenyl hydroxy comarnine derivatives for 48 h. Flow cytometry histograms analysis of the cells stained with PI and analyzed by flow cytometry assay. The percent of the cells in Sub-G1 peak were analyzed by *FlowJo* software. Profiles (A–D) are 6-Farnesyl oxi coumarin, 6-Geranyl oxi coumarin, 3-Farnesyl oxi coumarin, and 3-Geranyl oxi coumarin, respectively. ***P<0.001 versus control cells (0 µg/mL) regarding Prenyl hydroxy comarnine derivatives; **P<0.01 versus control cells (0 µg/mL) regarding Prenyl hydroxy comarnine derivatives.

chemical modification on them, as compared to the rest of the compounds, the richness of many plants of coumarin, and low toxicity[38]. Hydroxy coumarin derivatives participate in the proliferation process of human lymphocytes. To determine whether the induction of Hydroxy coumarin derivatives was relevant to the potentiation effect, experiments were performed in human lymphocytes. In lymphocytes, exposure to 0.75-200 µg/mL Hydroxy coumarin derivatives (200 µg/mL maximum concentration achievable) did not induce any antiproliferative effect (survival fraction>98%) in 24 hours. There was no effect on lymphocytes, which were incubated for 24 hours with Hydroxy coumarin derivatives. Then lymphocytes were incubated for 48 h and 72 h with 0.75-200 µg/mL Hydroxy coumarin derivatives. In the maximum concentration, a small potentiation effect was seen (survival fraction>98% for all fractions, except for 6-Farnesyl oxi coumarin). The survival fraction for 6-Farnesyl oxi coumarin in 48h and 72h with 200 µg/mL was ~75.5%, and 72.2%, respectively, and all concentration, failed to induce cytotoxicity (survival fraction ~97%). The effect of Hydroxy coumarin derivatives on lymphocytes as normal cells is presented in Fig. 3.

Coumarins, depending on the pattern of substitution and replacement of functional groups on the coumarin ring, act differently on different cancer cells[39]. Isoprenylated coumarin derivatives with systematic variations in the tail length (5, 10, or 15 carbons) and substitution position on the coumarin scaffold (at 3-, 6- or 7-position),

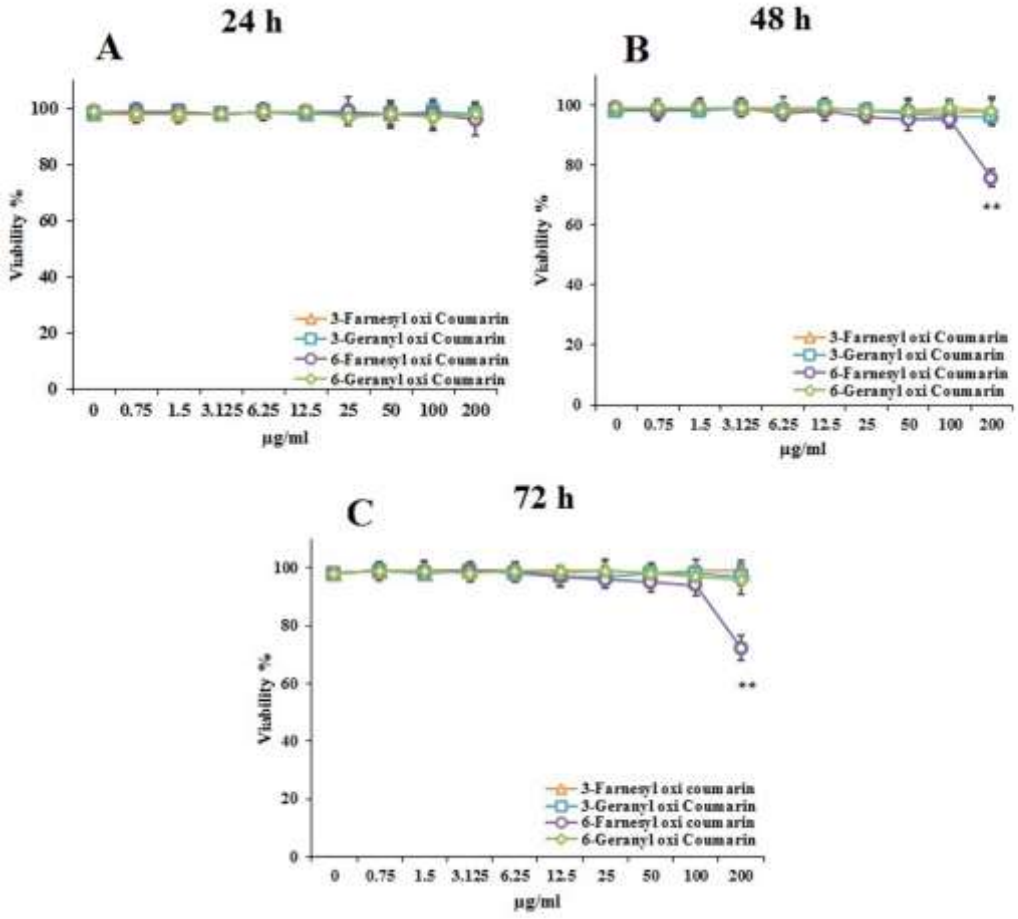

Figure 3. Cell survival rate of human lymphocyte evaluated by MTT. The cells at a density of 10^6cells/mL were cultured for 24 h, 48h and 72h in presence of different concentrations of Prenyl hydroxy comarnine derivatives (3-Farnesyl oxi coumarin , 3-Geranyl oxi coumarin, 6-Farnesyl oxi coumarin and 6-Geranyl oxi coumarin) (0–200 µg/mL). Data are presented as Mean ± SD of three independent experiments. After 48, 72 hour in 200 µg/mL concentration viability decreased. Profiles A, B and C are 24h, 48 and 72h respectively. **P<0.01 versus control cells (0 µg/mL) regarding Prenyl hydroxy comarnine derivatives.

causing synthesized coumarin derivatives of the coumarin [40]. According to this study, 6-substituted farnesylated ether coumarin derivative has the highest cytotoxic activity in the 24-hour incubation among the isoprenylated derivatives of coumarin[41].

In comparing all of them, Farnesyl oxi coumarin is stronger than Geranyl oxi coumarin and has significant biological activity. This study investigates the cytotoxic and proapoptotic effects of Prenyl hydroxy coumarin derivatives on neuroblastoma cells (N2A). In the author's opinion, this is the first report of the toxicity of Prenyl hydroxy coumarin derivatives (3-Farnesyl oxi coumarin, 3-Geranyl oxi coumarin, 6-Farnesyl oxi coumarin, and 6-Geranyl oxi coumarin) in this cancer cell line. Our data confirm that the Prenyl hydroxy coumarin derivative has cytotoxic effects against neuroblastoma cells, which is consistent with previous studies of other derivatives of coumarin, such as Furano

coumarins[41, 42], Dihydrofurano coumarins [43], Phenyl coumarins [44], and Bicoumarins [45]. Also, our data show that Prenyl hydroxy coumarin derivatives constitute a signifcant inhibitor of the proliferation of neuroblastoma cells (N2A).

It was found that among the Prenyl hydroxy coumarin derivatives, 3-Farnesyl oxi coumarin, and 6-Farnesyl oxi coumarin, have the most anti-proliferative activity after 24 h and 48 h, respectively. Previous results of other coumarin derivatives indicate that apoptosis is involved in the death of induced cancer cells [46]. Also, Hydroxy coumarin derivations treatment did not affect the proliferation of normal lymphocytic cells, indicating a degree of specificity for N2A cells.

Coumarin and its derivatives show cytotoxic effects in dose-dependent behaviors [48]. Cancer cells treated by coumarin and its derivatives show apoptotic properties such as nucleoplasm and cytoplasmic condensation, DNA fragmentation, formation membrane-bound apoptotic bodies, and loss of microvilli[48, 49].

The neuroblastoma cells were treated with different concentrations of Prenyl hydroxy coumarin derivatives (IC_{50}, low and more concentration) with a hypotonic phosphate–citrate buffer. The reported results indicate that after incubation of the neuroblastoma cells, DNA fragmentation, and small apoptotic components were observed. At present, the rate of apoptosis and DNA fragmentation is determined using PI staining of DNA fragmentation with flow cytometry (sub-G1 peak). In this staining with PI, the cells that have lost their DNA are less colored and appear to the left of the peak of the G1 peak[47-50]. This study shows that the Prenyl hydroxy coumarin derivatives prevent the proliferation of N2A tumor cells by interfering with apoptosis. These derivatives can be considered as promising chemotherapy agents for cancer treatment. At the end of 24h, 48h, and 72h incubation, Hydroxy coumarin derivatives did not show any cytotoxic effect on lymphocytes (Figure 3: A , B and C).

4. Conclusion

Neuroblastomas are cancers that start in early nerve cells (called neuroblasts) of the sympathetic nervous system, and they consist of 9% of childhood cancers .Apoptosis which is happening during development and aging is a homeostatic mechanism to preserve cell populations in tissues. Some anticancer drugs induce apoptotic pathways in cancer cell [14, 15]. Hydroxy coumarin derivatives have eligible significant anti-proliferative effects on neuroblastoma, renal, lung, and breast cancers, also leukemia[39, 51]. Notably, coumarin and its derivatives inhibit the growth of cancer cells, yet they do not affect normal cells [39, 48]. Therefore, these derivatives are more effective in cells whose growth or signaling pathways have changed, rather than normal cells. In the present study, five derivatives of Prenyl hydroxy coumarin were used in different concentration on N2a cell line. The results confirmed that 3-Farnesyl oxi coumarin and

6-Farnesyl oxi coumarin have more anti-proliferative and anti-apoptotic effects than 3-Geranyl oxi coumarin and 6-Geranyl oxi coumarin after 24 h and 48 h incubation with N2a cell line. These data were validated in high and low concentrations of IC50 through flow cytometry. These derivatives had no anti-proliferative and anti-apoptotic activates on normal lymphocytic cells. Then, findings indicated that these derivatives are important as anti-cancer agents.However, the precise molecular mechanisms of these Prenyl hydroxy coumarin derivatives are still unclear, but there is an ongoing research, that will soon provide a systematic mechanism of these derivatives.

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