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REAL-TIME ANALYSIS OF IMPEDANCE ALTERATIONS BY THE NEUROTOXICITY OF SCOPOLETIN ON SH-SY5Y NEUROBLASTOMA CELLS
SH-SY5Y NÖROBLASTOMA HÜCRELERİNDE SKOPOLETIN NÖROTOKSİSİTESİNE BAĞLI EMPEDANS DEĞİŞİKLİKLERİNİN GERÇEK ZAMANLI ANALİZİ

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ÖZ

Bitki kumarinleri, insan diyetinin önemli bileşenleridir ve birçoğunun kansere karşı koruyucu ve tedavi edici özelliklere sahip olduğu bilinmektedir. Doğal bir kumarin bileşeni olan skopoletinin birçok kanser hücresinin büyümesini engellediği gösterilmiştir. Bu çalışmada, insan nöroblastom hücreleri SH-SY5Y'ye karşı skopoletinin sitotoksik aktivitesi incelendi. Scopoletin aktivitesi, Sulphorhodamine B (SRB) testi ve xCELLigence sistemi ile değerlendirildi. Skopoletinin insan SH-SY5Y nöroblastom hücre canlılığını azalttığı gösterildi. Scopoletin hücre büyümesini inhibe etti ve IC50 değerleri 48 ve 72 saat için sırasıyla 91,82 ve 79,19 µM'dir. Bu çalışmadan elde edilen bulgular, skopoletinin SH-SY5Y hücreleri üzerindeki büyüme önleyici ve sitotoksik etkilerinin, nöroblastom kanseri için yeni tedavi rejimlerinin geliştirilmesinde önemli olabileceğini kanıtladı.

Anahtar kelimeler: SH-SY5Y, sitotoksisite, skopoletin, SRB, xCELLigence.

ABSTRACT

Plant coumarins are important components of the human diet and a number of them are considered to possess chemo-preventive and therapeutic properties against cancer. Scopoletin, a natural coumarin component has been shown to inhibit the growth of many cancer cells. In this study, the cytotoxic activity of scopoletin was studied against human neuroblastoma cells SH-SY5Y. The activity of scopoletin was evaluated by Sulphorhodamine B (SRB) assay and xCELLigence system. It was demonstrated that scopoletin reduced human SH-SY5Y neuroblastoma cell viability. Scopoletin inhibited the growth of the cell line and the IC50 values were 91.82 and 79.19 µM for 48-h and 72-h, respectively. The findings from this study demonstrated that the growth inhibitory and cytotoxic effects of scopoletin on SH-SY5Y cells may prove essential in the development of novel treatment regimens for neuroblastoma cancer.

Keywords: Cytotoxicity, Scopoletin, SH-SY5Y, SRB, xCELLigence.

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INTRODUCTION

There is increasing interest in plant products for using them against the malignant progression of neoplastic diseases in the world (1,2). Phytochemicals that have multiple pharmacologic activities provide important protection concerning different types of cancer (3-5). Increasing interest has occurred in yielding a scientific base for the utilization of phytochemicals as a prophylactic method for those at high risk of cancer. Scopoletin (6-methoxy-7-hydroxy coumarin) a natural anticarcinogenic and apoptotic coumarin component. Between plant-based products, coumarins constitute a common group of secondary metabolites (6). They have been shown to demonstrate a broad variety of bioactivities, like anticancer activity. Scopoletin has been shown to limit the growth of cancer cells (7-9). There are drug-likeness and medicinal chemistry results that confirm Scopoletine's possibility of being a drug (7). Neuroblastoma, the solid extracranial neoplasm, is characterized by neuroblastic tumor cells resembling embryonal sympathetic neuroblasts of the neural crest and is often arising from extracranial malignancy in childhood (10). Though multimodal therapeutic attempts such as chemotherapy, radiotherapy, and immunotherapy are used, the survival rate for patients with malignant neuroblastoma is low (11). Hence, there is a requirement to develop new agents to evolve therapy results in this risky population. Natural products are of specific attention with the improvement of novel attempts at chemotherapy (12). The SH-SY5Y cell line originates from human neuroblastoma. SH-SY5Y cells can be induced to differentiate into mature neurons, which display a different morphology and are with easy detected (13). Scopoletin's anticancer effects have not been studied against neuroblastoma cancer cells SH-SY5Y. Therefore, in this study, the SH-SY5Y cell line was used to detect the effect of scopoletin on neuroblastoma cell lines in a real-time way.

MATHERIAL AND METHODS

Chemical and Reagents

Scopoletin (No: 38332) was acquired from Sigma Chemical Company. Dimethyl sulfoxide (DMSO) (No: A3672) was purchased from Applichem. Dulbecco's modified eagle's medium (DMEM) (No: 01-052-1A) and penicillin/streptomycin solution (No: 03-031-1C) were acquired from Biological Industries. Fetal bovine serum (FBS) (No: S0115) was acquired from Biochrom. Trypsin-EDTA (No: T3924) was supplied from Sigma. Sulforhodamine B (SRB) sodium salt (No: sc-253615A) was purchased from Santa Cruz Biotechnology.

Cell Culture

Human neuroblastoma cell line SH-SY5Y was supplied from the American Type Cell Collection (No: CRL-2266™ ATCC, USA). The cell line was grown in DMEM with 10% (v/v) FBS. The cell line was maintained at 37°C under humidified air with 5% CO2. Trypsin 0.25% EDTA was used in a subculture. Scopoletin was dissolved in DMSO. The concentration of DMSO was below 0.1% in the medium.

Cell Viability Analysis via Sulphorhodamine B Assay

The neurotoxic effect of scopoletin on SH-SY5Y cell viability was assessed by the method of SRB assay (27,28). Scopoletin was dissolved in DMSO. 10.000 cells/well seeded. Then cells treated with scopoletin (3-300 µM). The cells were fixed in 10% (w/v) of trichloroacetic acid (TCA) during 1 h at 4 °C. The cell monolayers were stained 0.5% (w/v) SRB solution for 30 min; then, the excess dye was decreased with 1% (v/v) acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution for optical density (OD) determination at 510 nm utilizing a microplate reader (Biotek Synergy HT). Cell viability was defined as a percentage of the control values.

Monitoring Neurotoxicity Using xCELLigence RTCA

The xCELLigence system is a method for measuring cell viability following the manufacturer's instructions. The xCELLigence system forms the RTCA (ACEA Biosciences Inc., San Diego, CA, USA), the RTCA single plate (SP) station, the RTCA computer with integrated software, and a disposable E-plate. The experimental principle of the growth profile in real-time is related to measuring the variation in the adhesive features when attached to the microelectrode-coated surface of the cells in the E-plate. Impedance reading delivers time quantitative data on the contemporary state of cells. For example viability, morphology, cell number, and motion. As in classical end-point experiments, measurements are made without using labels or chemicals. The software of the instruments authorizes real-time monitoring, data visualization and analysis functions (14,29). SH-SY5Y cells seeded to E-plate of xCELLigence RTCA SP system. In brief, 10.000 cells/100 µL per well were added to obtain the cell profile of the SH-SY5Y. xCELLigence system computes the impedances parameter named "Cell Index (CI)". The CI values of SH-SY5Y were employed to analyze cell profile contingents on proliferation and viability over 96 h. The RTCA software applies the "sigmoidal dose-response equation" curve to the experimental data points and computes the logarithmic half-max effect of the concentration (log (IC50)) at a presented time point relative to the concentration. A 50% reduction in CI relative to the control CI value (100%) is expressed as log IC50. (30).

Statistical Analysis

All calculations from xCELLigence were obtained using the RTCA-integrated software of the xCELLigence system. Statistical analysis of the results obtained was carried out by GraphPad Prism Software Version 8.1.0 (La Jolla, CA, USA) using to compare differences in values between the control and experimental group. The results generated are presented as the Median (IQR).

Results were analyzed with Kruskal-Wallis test. P-values determined using the Dunnett's and least significant difference post hoc test. Differences among medians were considered statistically significant when the value was less than 0.05. *P<0.05, **P<0.01, and
***P<0.001

RESULTS

Neurotoxic Effect of Scopoletin

To assess the therapeutic effectiveness of scopoletin in neuroblastoma cell lines, the viability of SH-SY5Y cells was determined. The cell viability in the presence of scopoletin was analyzed by SRB assay. Five various concentrations of scopoletin (3, 10, 30, 100, and 300 µM) were chosen to evaluate the neurotoxic impact of scopoletin on the viability with a broad spectrum. Scopoletin treatment at 3, 10, and 30 µM concentration did not significantly alter cell viability for 72 hours (Figure I, Table I)

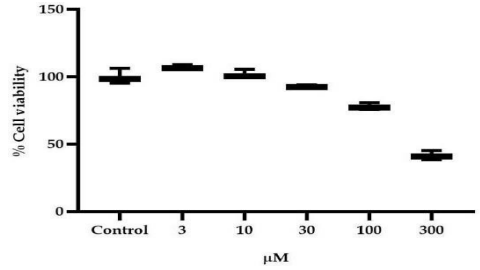


Figure I. The cytotoxic effect of scopoletin was shown for 72 h. *** P < 0.001 compared with the untreated control.

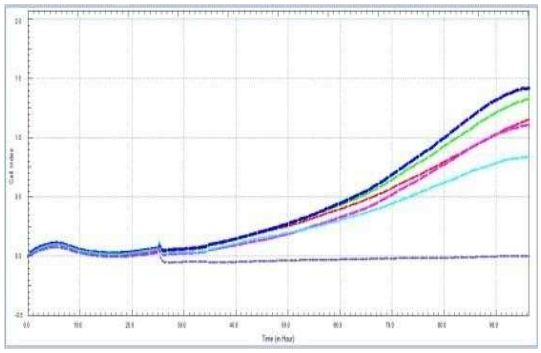
The cell viability decreased at concentrations above 30 µM. The subsequent attempt was to work with a broad range of concentration.In the SH-SY5Y cell line, the cell viability decreased indistinctly at 30 µM and significantly (***P<0.001) at 100 and 300 µM concentrations (Figure I).

Table I. Statistically results for the cytotoxic effect of scopoletin for 72 h.

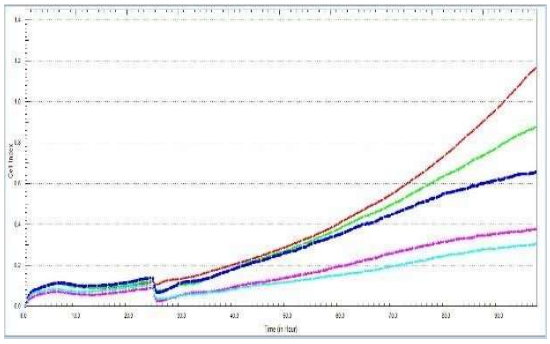
Variables	Median	I Q R	P
Control vs. 3	106.843	2.043	0.082
Control vs. 10	101.265	3.649	0.914
Control vs. 30	92.779	1.157	0.084
Control vs. 100	77.680	2.815	<0.001
Control vs. 300	41.191	3.672	<0.001

Monitoring of Scopoletin's Toxicity in Real-Time Using xCELLigence System

The dynamic proliferation of SH-SY5Y cells was monitored for 72 h. When the growth profile of SH-SY5Y cells was examined, the CI values were not very high. Many factors affect the CI value as cell size, morphology, and cell-substrate adhesion quality (14,15) and it was thought to occur depending on them.



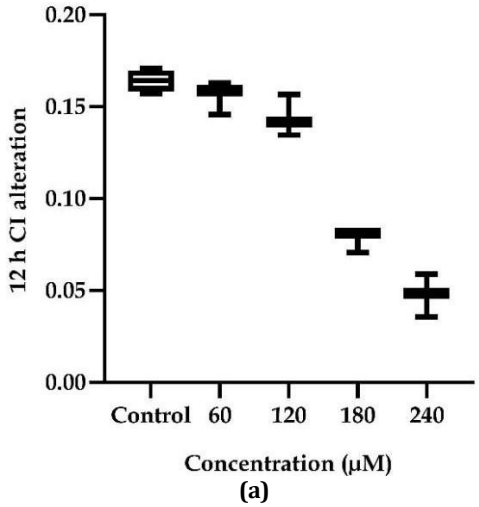
(a)



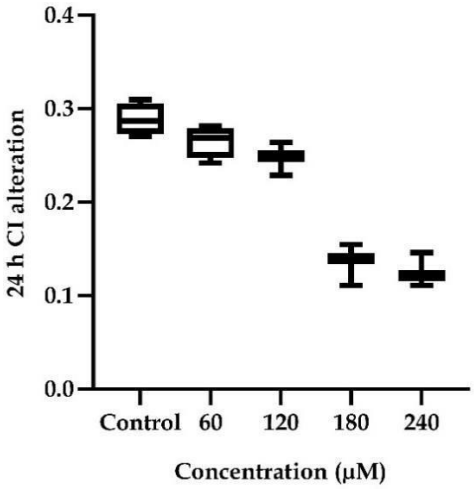
(b)

Figure II. Concentration and time-dependent neurotoxic effect of scopoletin on SH-SY5Y determined by xCELLigence system: (a) Cells were treated with scopoletin (3-300 µM) for 72 h. Lines: Red; control, green; 3 µM, indigo; 10 µM, pink; 30 µM, turquoise; 100 µM, purple; 300 µM. (b) Cells were treated with scopoletin (60-240 µM) for 72 h. Lines: Red; control, green; 60 µM, indigo; 120 µM, pink; 180 µM, turquoise; 240 µM. The 4 repeated measurements were calculated from the CIs.

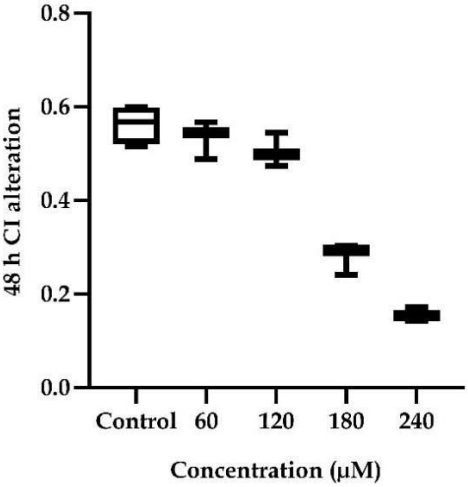
To monitor and verify the reliability of SRB test results, the cell viability was assessed utilizing the RTCA in parallel with the assay given in section 2.1. Test results of the RTCA indicate a parallel result for the concentrations of 3, 10, 30, 100, and 300 µM when assessed with the SRB test results (Figure I and IIa). Four different concentrations (60, 120, 180, and 240 µM) of scopoletin were chosen to evaluate the effect of the compound on cell viability by xCELLigence (Figure IIb). Results expressed that scopoletin inhibited proliferation of SH-SY5Y in a concentration- and time-dependent manner when treated at 60-240 µM concentrations, with IC50 values of 91.82 µM and 79.19 µM at 48 and 72 hours, respectively. As the cells reacted to scopoletin, changes in CI alterations were calculated with the RTCA SP instrument for 72 hours. It was seen that a correlation exists between the results generated in section 2.1. using the real-time measurement and in section 2.2. using the end-point method, SRB assay. The concentration-response curves and the viability of the cells at the specified time points are shown in Figures IIa and b. Control groups indicated normal cell growth in electronic microwells. As shown in Figures IIa and b, the CI values of the treated cells reduced from upper than 30 µM in a concentration-dependent way. Control's CI values continued to rise to 72 hours post-treatment. In the cell line, the CI alterations declined at all concentrations compared with the control (Figure III, Table III). Scopoletin has an anti-proliferative effect at concentrations of 60 and 120 µM (Figure IIIa, b, and c); the cell viability declined at 60, 120, 180, and 240 µM in a concentration-dependent way (Figure IIIa, b, c, and d). RTCA viability assay results showed scopoletin reduced viability by at least 70% at concentrations of 180 and 240 µM (Figure IIIa, b, c, and d).



(a)



(b)



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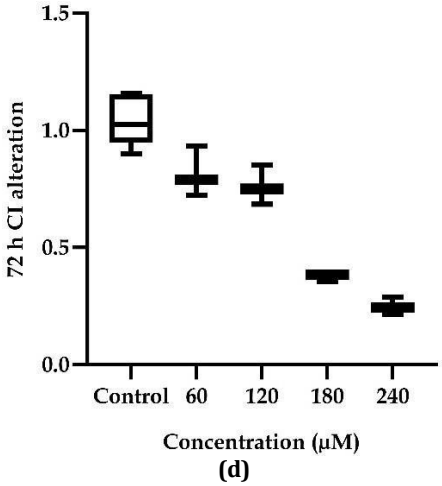

(d)

Figure III. CI obtained from the xCELLigence system. Correlation between CI and the scopoletin neurotoxic effects using the RTCA system. The compound was applied with different concentrations for (a) 12 h, (b) 24 h, (c) 48 h, and (d) 72 h incubation of the SH-SY5Y cell line., (*P<0.05, **P<0.01, and ***P<0.001) crosschecked with the untreated control. CI: Cell index

Tablo II. Statistically results for the CI.

Variables	Median	IQR	P
12 h			
Control vs. 60	0.157	0.010	0.274
Control vs. 120	0.143	0.013	0.040
Control vs. 180	0.080	0.007	<0.001
Control vs. 240	0.049	0.013	<0.001
24h			
Control vs. 60	0.269	0.024	0.701
Control vs. 120	0.248	0.019	0.183
Control vs. 180	0.138	0.025	<0.001
Control vs. 240	0.124	0.020	<0.001
48 h			
Control vs. 60	0.539	0.046	0.011
Control vs. 120	0.502	0.039	0.003
Control vs. 180	0.286	0.039	<0.001
Control vs. 240	0.156	0.016	<0.001
72 h			
Control vs. 60	0.802	0.118	0.602
Control vs. 120	0.757	0.091	0.051
Control vs. 180	0.381	0.022	<0.001
Control vs. 240	0.247	0.039	<0.001

DISCUSSION

An embryonal cancer of the autonomic nervous system is neuroblastoma that is generally diagnosed in the first year of life, with a prevalence of 1 out of 100,000 children (16). The treatments for neuroblastoma contain chemotherapy, surgery, radiotherapy, stem cell transplantation, and immunotherapy (12). Scopoletin (6-methoxy-7-hydroxycomarin) is a constituent of the phytoalexin group isolated from many plants and is an important coumarin derivative that occurs in various plants (17). There are many studies in the literature showing that scopoletin exhibits significant and important pharmacological activities (18-21). Although the potential protection of scopoletin is known to all, some literature findings indicate that it has useful effects such as anti-angiogenic (18), anticancer (19), apoptotic (17), anti-inflammatory (20), and anti-allergy (21) subjecting on dosage and use conditions. However, there are few studies in the literature investigating the effect of scopoletin on neuroblastoma cells (22-24). The present study aimed to evaluate the cytotoxic activity of scopoletin against SH-SY5Y human neuroblastoma cells in vitro. In the present study, scopoletin has shown a neurotoxic effect on SH-SY5Y neuroblastoma cells. The anti-cancer mechanism of scopoletin on all types of cancer has not been fully detailed yet. SRB results showed that scopoletin had an anti-neuroblastoma effect. The neurotoxic impacts of the compound, the 50% inhibitory concentrations (IC50) were computed at the end of 48-h and 72-h treat with xCELLigence. Scopoletin IC50 values were calculated as 91.82 and 79.19 μM, respectively. Scopoletin has shown its cytotoxic activity on different cancer cell lines (7,8). Recently, the pharmacological mechanisms of the anticancer effect of scopoletin have been also recorded. In literature, scopoletin displayed antitumor effects on human prostate tumor cells and leukemia cells by way of stimulating cell cycle arrest and initiating apoptosis (22,23), and also showed significant therapeutic potentials against 7, 12-dimethylbenz(α)anthracene (DMBA)-induced skin cancer in mice (24). More importantly, scopoletin also lacks cytotoxicity to normal cells. In a study, scopoletin had anti-non-small cell lung carcinoma (NSCLC) effect. But it had no toxic and side effects on normal lung epithelial cells. Also reduced the activation of epidermal growth factor receptor (EGFR), thereby inhibiting the RAS-RAF-MEK-ERK pathway and PI3K/AKT pathway, leading to apoptosis of NSCLC and inhibiting its proliferation. (31). In addition, numerous studies have witnessed the effective anti-tumor activity of this compound and derivate by modulating key components of cell signaling pathways that control important features of this disease such as cell proliferation, survival, apoptosis, autophagy, migration. metastasis, such as cell proliferation, survival, apoptosis, migration, and metastasis (31-34).

According to a study, the neurotoxicity of scopoletin was determined in SH-SY5Y cells using MTT assay. The treatment with concentrations up to 20 μM did not affect neuronal viability. In our study, it was found that no cytotoxic effect was observed at the determined concentrations (35). In another study, scopoletin lessened rotenone-induced apoptosis in SH-SY5Y cells through preventing oxidative injury. The concentration of 30 μM scopoletin was used for pre-treated due to maximum viability was achieved at that concentration. It was found markedly upregulate DJ-1, which then promoted the nuclear translocation of Nrf2 and transactivation of antioxidant genes. Additionally, scopoletin prevents the nuclear exportation of Nrf2 by reducing the levels of Keap1 and thereby enhancing the neuronal defense system. Overall, our findings suggest that scopoletin acts through DJ-1-mediated Nrf2 signaling to protect the brain from rotenone-induced oxidative stress and Parkinson Disease's (36). Nevertheless, its weak antitumor activity in vitro and in vivo and a high elimination rate in vivo limited its clinical application. For this reason, scopoletin is considered as an optimal main compound for designed and synthesized plenty of derivatives (25,26,32). Scopoletin can target multiple molecular targets to attenuate disease progression (34). When these results and the literature are evaluated together, the mechanism of neurotoxicity in SH-SY5Y cells has been opened in future studies.

CONCLUSION

Consequently, scopoletin is an effective coumarin with cytotoxic features. Scopoletin inhibits the growth of neuroblastoma cells. It may demonstrate advantages in treating neuroblastoma and therefore highlights the importance of scopoletin-based molecular mechanism evaluations.

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Conflict of interest statement

"The authors declared no conflict of interest"

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