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Cytotoxic and antiproliferative effects of hellebrin on breast and lung cancer cells

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ABSTRACT

This study aimed to investigate the impact of hellebrin on human lung cancer cell (H1299) and breast cancer cell (MCF-7) lines over time. The viability of various concentrations of hellebrin (100 nM-400 nM) on two cancer cell lines was measured using the MTT method. The cellular proliferation over time was examined using xCELLigence real-time cell analysis (RTCA). The results showed a significant decrease in cell viability with increasing concentrations of hellebrin in both cancer cell lines compared to the control group ($p<0.001$). While the viability of both cancer cells decreased after a certain period of hellebrin application, the viability of the control groups increased over time. These findings indicate that hellebrin has high antiproliferative and cytotoxic effects on breast and lung cancer cells. Consequently, this study suggests that hellebrin may have potential as a treatment for other cancers, especially lung cancer, and further in vitro and in vivo experiments are needed to investigate this possibility.

Hellebrin'in meme ve akciğer kanseri hücreleri üzerinde sitotoksik ve antiproliferatif etkileri

ÖZET

Bu çalışmada, hellebrinin insan akciğer kanseri hücresi (H1299) ve meme kanseri hücresi (MCF-7) hatları üzerindeki etkisinin zaman içinde araştırılması amaçlanmıştır. Hellebrin'in (100 nM-400 nM) çeşitli konsantrasyonlarının iki kanser hücre hattı üzerindeki canlılığı, MTT yöntemi kullanılarak ölçüldü. Zaman bağlı hücresel çoğalma, xCELLigence gerçek zamanlı hücre analizi (RTCA) kullanılarak incelenmiştir. Sonuçlar, kontrol grubuna kıyasla her iki kanser hücre hattında artan hellebrin konsantrasyonları ile hücre canlılığında önemli bir düşüş gösterdi ($p<0.001$). Belli bir süre hellebrin uygulamasından sonra her iki kanser hücresinin canlılığı azalırken, kontrol gruplarının canlılığı zamanla arttı. Bu bulgular, hellebrin'in meme ve akciğer kanseri hücreleri üzerinde yüksek antiproliferatif ve sitotoksik etkilere sahip olduğunu göstermektedir. Sonuç olarak, bu çalışma hellebrin'in diğer kanserler, özellikle akciğer kanseri için bir tedavi potansiyeline sahip olabileceğini ve bu olasılığı araştırmak için daha fazla in vitro ve in vivo deneylere ihtiyaç olduğunu düşünülmektedir.

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1. Introduction

In general, the most common type of cancer seen in countries are determined by the socio-economic conditions of the countries, which also affected the treatment period, recovery rate and death rate of the disease (1-3). Breast cancer (especially in women) and lung cancer-which are among the most common cancers around the world-are also at the top of cancer-related deaths (4, 5). Although methods such as surgical intervention, radiotherapy, chemotherapy and hormone therapy are widely used in cancer treatment around the world, there is a need for the development of easy-to-access and effective drugs for cancer treatment (2). Especially in recent years, it has been tried to reduce the mortality rate by using cytotoxic agents directed to cells (target) in cancer treatment (6).

In recent years, natural compounds obtained from Helleborus (family Ranunculaceae) species have been used in the treatment of various diseases in human and veterinary medicine (7). Hellebrin, obtained from Helleborus species, is a cardiotonic glycoside that has the structure of bufadienolide (7, 8). Bufadienolides are steroids of vegetable or animal origin with an unsaturated six-membered lactone ring (α-pyrone ring) at the C17 position and have cardiotonic, antiviral, anti-inflammatory, antimicrobial, and anti-cancer effects (9-11). In cancer studies, bufadienolides show various effects, such as inducing apoptosis and autophagy in cells (12, 13), and inhibiting epithelial-mesenchymal transition (14-16). Various bufadienolides such as hellebrin, hellebrigenin and bufatalin have been reported to inhibit various human and mouse cancer cells at various doses (72nd hour MTT results) in *in vitro* studies (8, 17). In this study, it was aimed to show the effect of hellebrin on human lung cancer cell (H1299) and breast cancer cell (MCF-7) lines depending on time.

2. Material and Methods

Hellebrin (Cayman, USA) was prepared by dissolving in medium. MCF-7 and H1299 cancer cell lines were obtained from Kirikkale University Scientific and Technological Research Application and Research Center (KUBTUAM) cell culture collection. This study was carried out in the KUBTUAM laboratory.

Viability test

The MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Ambresco-Life Science, USA] test was used to determine the cytotoxicity of hellebrin. After counting MCF-7 and H1299 cells in the hemocytometer (Invitrogen-Countess, USA), a plate (96-well plate) was seeded with 1.10^4 cells in each well. After the cells were incubated for 24 hours, different concentrations of hellebrin (100 nM, 200 nM, 250 nM, and 400 nM) were applied. In addition, the medium was used as a control. After 24 hours, the medium was discarded and 50 µL of the MTT (1 mg/mL) solution was applied to the wells. Two hours after MTT application, 100 µl of isopropanol (Sigma, Germany) was added to the wells and read in an ELISA (Biotek, USA) device at a wavelength of 570 nm. The MTT test (repeated the MTT test 3 times) was performed in triplicate according to the TS EN ISO 10993-5 standard (18). The cell viability calculation was made according to the following formulation.

Viability %: [concentrations of hellebrin (the average value of absorbance)/control (the average value of absorbance)] x 100.

Real time cell analysis system (xCELLigence) test

After counting MCF-7 and H1299 cells, the 96-well e-plate was seeded with 5.10^3 cells in each well, and the e-plate was placed in RTCA-SP (Roche, Germany). After the cells were incubated for 24 hours, concentrations of hellebrin (100 nM, 200 nM, 250 nM, and 400 nM) were applied. The medium was used as a control. RTCA-SP device made real-time impedance measurements every 10 minutes and was monitored for approximately 68 hours. Graphs showing the time-dependent change of MCF-7 and H1299 cells were obtained (19).

Statistical evaluation

Data obtained by the MTT test were given as arithmetic mean ± standard error (SE). SPSS program (PASW Statistics for Windows, version 18.0. Chicago, USA) was used to determine the statistical difference between the

groups. The significance control of the difference between groups was determined by the Tukey test (post hoc). P value < 0.05 was considered statistically significant. The IC_{50} value was determined using GraphPad Prism (Software ver. 6) according to the MTT assay results.

3. Results

Viability test results

The effect of hellebrin concentrations on MCF-7 and H1299 cell viability according to the MTT test is given in Table 1. The statistical difference between hellebrin concentrations and the control group (medium) were given in Figure 1 ($p<0.001$). It was determined that cell viability decreased from low to high concentration of hellebrin on both cancer cell lines. Hellebrin has an IC_{50} value of about 1391 nM on MCF-7 cells, while an IC_{50} value on H1299 cells is about 65.1 nM. Specifically, hellebrin concentrations were found to be more cytotoxic on H1299 cells compared to MCF-7 cells.

Table 1: Effect of control and hellebrin concentrations on MCF-7 and H1299 cells
Tablo 1: *Kontrol ve hellebrin konsantrasyonlarının MCF-7 ve H1299 hücreleri üzerindeki etkisi*

Concentration (nM)	**MCF-7**	**H1299**
	Viability %	*Viability %*
400	69.87±4.03[a]	39.78±1.87[a]
250	70.99±2.79[a]	40.35±3.08[a]
200	80.69±2.93[a]	43.93±1.34[a]
100	84.07±3.85[a]	47.54±5.17[a]
Control	100±1.88[b]	100±5.19[b]

[a,b] *Different letters in the same columns are important (mean ± SE, p<0.001).*

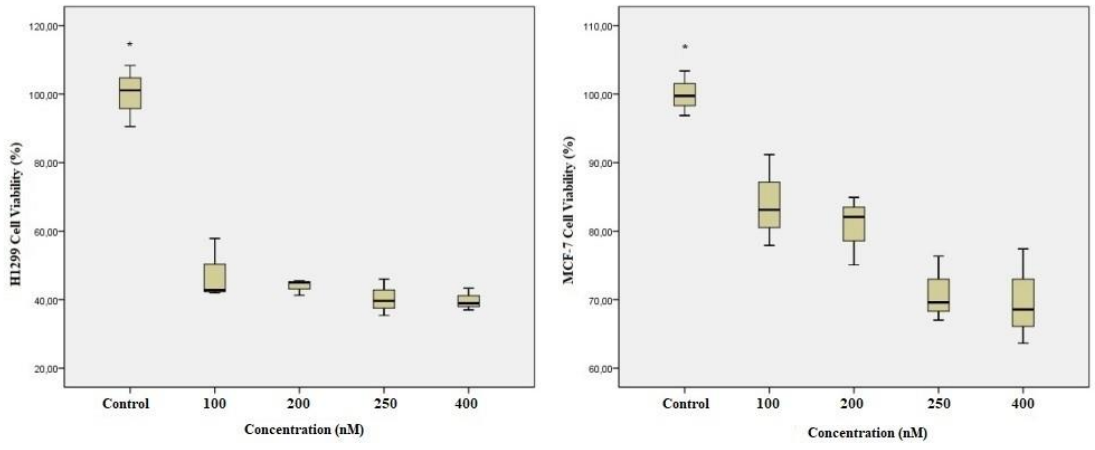

Figure 1: Effect of control and hellebrin concentrations on MCF-7 and H1299 cells, mean ± SE, ($p<0.001$)
Şekil 1: *Kontrol ve hellebrin konsantrasyonlarının MCF-7 ve H1299 hücreleri üzerindeki etkisi, ortalama ± SE, (p<0.001)*

xCELLigence system cell proliferation results

The effects of various concentrations of hellebrin on MCF-7 and H1299 cells were evaluated with the xCELLigence system, which provides information on the viability and proliferation of cells in a time-dependent manner by means of micro-electronic biosensors. The temporal cell proliferation graphs of hellebrin and control groups applied on MCF-7 and H1299 cells are given in Figures 2 and 3. Approximately 68 hours were monitored

from the seeding of cancer cells to the e-plate, including hellebrin concentrations and control group administration. After a certain period of time after Hellebrin application, it was observed that the viability of both cancer cells was greatly reduced. On the other hand, in the control groups (medium) of both cancer cell lines, it was observed that the cells increased over time.

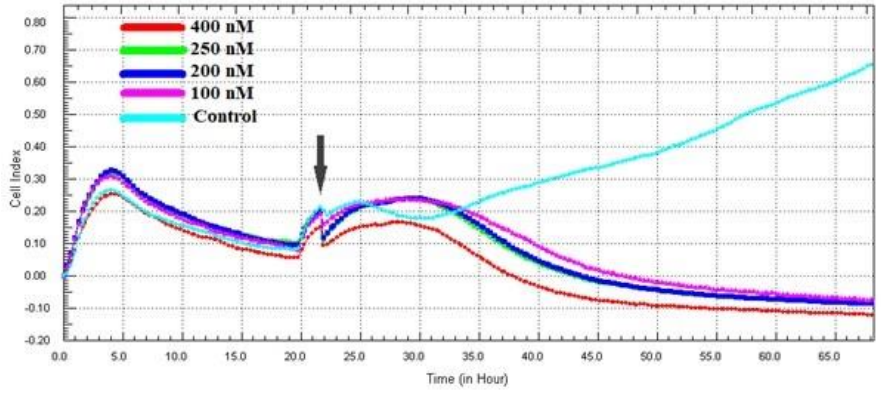

Figure 2: Time-dependent graph of MCF-7 cells with control and hellebrin concentrations applied
Şekil 2: *Kontrol ve hellebrin konsantrasyonları uygulanmış MCF-7 hücrelerinin zamana bağlı grafiği*

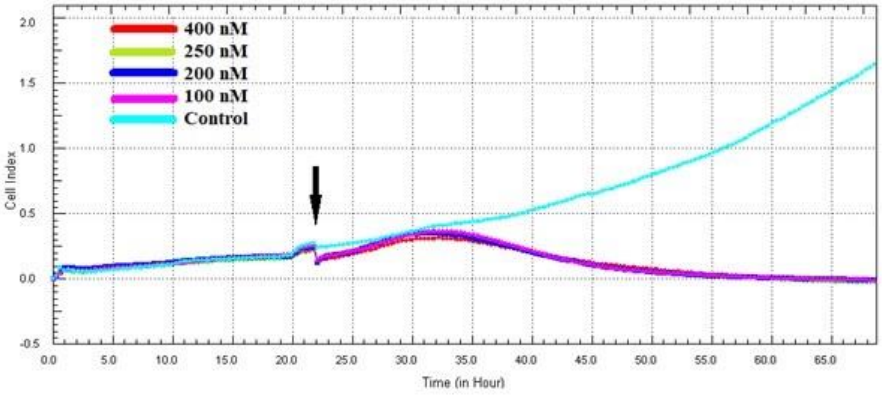

Figure 3: Time-dependent graph of H-1299 cells with control and hellebrin concentrations applied
Şekil 3: *Kontrol ve hellebrin konsantrasyonları uygulanmış H1299 hücrelerinin zamana bağlı grafiği*

4. Discussion and Conclusion

Despite the developing technology in the world, both time and financial losses are common in the development of new drugs as an alternative to traditional cancer treatments. Alternative treatment methods or drugs should be developed to minimize these losses and to provide drugs with wide confidence intervals (20). For this reason, this present study was conducted to evaluate the effectiveness of hellebrin on cancer cells [human lung cancer cell (H1299) and breast cancer cell (MCF-7)].

xCELLigence RTCA is a system used to determine time-dependent parameters such as cellular proliferation, toxicity (cytotoxicity), adhesion, and migration (21, 22). On the other hand, MTT is a widely used cytotoxicity method to determine cell viability and proliferation (23). This method determines cellular viability/cytotoxicity by reduction of tetrazolium salts to purple-blue colored formazan (insoluble in water) in lysosomal and endosomal sections, especially in mitochondrial enzymes (24-27). It is stated that the cellular toxicity that occurs is mainly due to mitochondrial damage (28).

In the present study, cellular toxicity of cancer cell lines to which hellebrin concentrations were applied increased to higher values in the xCELLigence RTCA system at earlier times compared to MTT results (Table 1) (Figures 2 and 3). According to the xCELLigence RTCA system, hellebrin significantly decreased cellular viability at approximately

the 16th hour on both cancer cell lines (Figures 2 and 3). On the other hand, MTT results show that cellular viability decreases to a certain extent approximately 26 hours after hellebrin application. When the results of the two tests are compared, hellebrin may have caused morphological changes in cancer cell lines, especially before mitochondrial damage. The reason for this is that the xCELLigence RTCA system cell index (CI) determines the electrical impedance value measured according to the growth, shrinkage, death, adhesion, and morphological changes of the cells on the gold-plated e-plate. In other words, the cell index increases due to the increase in the number of adherent cells on the e-plate electrode surface. In addition, this situation is affected by the increase in the adhesion area of the cells with the growth of the cells. As a result, the decrease in the number of cells, shrinkage of the cells, and decrease/loss of their adhesion capacity, which are the causes of a decrease in the cell surface on the electrode contact surface, cause a decrease in the cellular index (29). According to xCELLigence and MTT results, it is thought that hellebrin may cause cells to shrink or lose their adhesion ability before cellular death. Over time, this causes death in cancer cell lines.

Generally, during apoptosis, the connection of cells with neighboring cells decreases and shrinks (the cell shrinks), while in necrosis, the cells absorb fluid and swell. As a result of swelling of the cells in necrosis, the integrity of the cell membrane is disrupted, causing the cell contents to spread to the surrounding tissues. In comparison, this does not occur in apoptotic cells (30). As mentioned above, according to the results of xCELLigence (Figures 2 and 3) and MTT (Table 1), hellebrin may induce apoptosis by causing the cells to shrink or decrease/lose adhesion ability before the death of cancer cells. In this way, it is thought that hellebrin may prevent the contents of cancer cells from spreading to the surrounding tissues. In addition, studies have reported that bufadienolides such as bufarenogin and arenobufagin induce apoptosis in cells (12, 31). The idea that hellebrin, which is in the family of bufadienolides such as bufarenogin and arenobufagin, promotes apoptosis in breast and lung cancer cells, is consistent with the studies performed (12, 31). In addition, Daniel et al. (32) reported in their study that hellebrin causes apoptosis (eighth hour) at a higher rate than necrosis on Jurkat T lymphoblasts. This present study supports the study of Daniel et al. (32) and also showed a change in the temporal cell index.

As a result, hellebrin has a high rate of antiproliferative and cytotoxic effects on both breast and lung cancer cells. Especially demonstrated in the present study, the presence of antiproliferative and cytotoxic effects of hellebrin at lower concentrations on lung cancer cells may be guided in other cancer treatments, especially lung cancer, by conducting new *in vitro* and *in vivo* experiments.

Conflict of Interest

The author declared that there is no conflict of interest.

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Ethical Statement

An ethical statement was received from the authors that the data, information, and documents presented in this article were obtained within the framework of academic and ethical rules and that all information, documents, evaluations and results were presented in accordance with scientific ethics and moral rules.

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