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Determination Of Cytotoxic Effect Of Amygdalin In DLD-1 Cell Line and Anticytotoxic Effect In CCD-18CO Cell Line

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Research Article

History

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ABSTRACT

Objective: Amygdalin, which is part of the aromatic cyanogenic glycoside group, is found in plant seeds such as apricot, peach, plum, apple, pear, and cherry. It has been shown that amygdalin has anti-tumor properties against many cancers such as colon, breast, and lung cancer. This study aimed to determine the cytotoxic and anticytotoxic effects of amygdalin in human colon cancer cells (DLD-1) and normal colonic epithelium (CCD-18Co) using the MTT (3-(4,5-dimethylthiazol-2-YL)-2,5-diphenyltetrazolium bromide) test.

Materials and Methods: DLD-1 and CCD-18Co cells were grown in flasks containing Roswell Park Memorial Institute-1640 and Eagle's Minimum Essential Medium, respectively. Both cell groups were treated with amygdalin concentrations of 100, 50, 25, 12.5, 6.25, 3.125, and 1.56 mM for 24 hours. Then, 20% MTT dye was added to the wells of the aspirated plates and incubated for 3 hours. After the reaction was stopped with pure Dimethyl Sulfoxide (DMSO) at the end of the period, the absorbance values of the plates were read spectrophotometrically at a wavelength of 570 nm.

Results: The percent viability values for the DLD-1 cell line were found to be between 48.3-71.6% and the IC50 value was calculated as 74.03 mM. The viability values for the CCD-18Co cell line after the amygdalin treatment ranged from 101.6 to 117.9%.

Conclusion: While amygdalin showed a cytotoxic effect in the DLD-1 cell line, it showed an anticytotoxic effect in the CCD-18Co cell line. In our study, it was determined that amygdalin decreased the viability of DLD-1 cancer cells in a dose-dependent manner and did not show cytotoxic effects on CCD18-Co normal epithelial cells. More comprehensive controlled clinical trials are needed to demonstrate the feasibility of using amygdalin in combination with other anti-tumor drugs and to develop the artificial synthesis of the active ingredients in amygdalin in order to increase the anti-tumor activities of these drugs.

Keywords: Amygdalin, Cancer treatment, Cell culture, Colon cancer, Vitamin B17

Amigdalinin DLD-1 Hücre Dizisindeki Sitotoksik Etkisinin ve CCD-18CO Hücre Hatında Antikitotoksik Etkisinin Belirlenmesi

Süreç

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ÖZ

Amaç: Aromatik siyanojenik glikozitler grubunda yer alan amigdalin kayısı, şeftali, erik, elma, armut, kiraz gibi bitki tohumlarında bulunur. Amigdalinin kolon, meme ve akciğer kanseri gibi birçok kansere karşı anti-tümör özellikte olduğu gösterilmiştir. Bu çalışma, amigdalinin insan kolon kanseri hücrelerinde (DLD-1) ve normal kolon epitelinde (CCD-18Co) sitotoksik ve anti-sitotoksik etkilerini MTT (3-(4,5-dimetiltiyazol-2-YL)-2,5 difeniltetrazolyum bromür) testi kullanarak belirlemeyi amaçlamıştır.

Gereçler ve Yöntemler: DLD-1 ve CCD-18Co hücreleri, sırasıyla Roswell Park Memorial Institute-1640 ve Eagle's Minimum Essential Medium içeren şişelerde büyütüldü. Her iki hücre grubu da 24 saat boyunca 100, 50, 25, 12.5, 6.25, 3.125 ve 1.56 mM'lik amigdalin konsantrasyonları ile muamele edildi. Daha sonra aspire edilen plakların kuyucuklarına %20 MTT boyası eklendi ve 3 saat inkübe edildi. Süre sonunda saf Dimetil Sülfoksit (DMSO) ile reaksiyon durdurulduktan sonra plakların absorbans değerleri 570 nm dalga boyunda spektrofotometrik olarak okundu.

Bulgular: DLD-1 hücre dizisi için canlılık yüzdesi değerleri %48,3-71,6 arasında bulundu ve IC50 değeri 74,03 mM olarak hesaplandı. Amigdalin uygulamasından sonra CCD-18co hücre hattı için canlılık değerlerinin %101,6 ila %117,9 arasında değiştiği görüldü.

Sonuç: Amigdalin, DLD-1 hücre hattında sitotoksik etki gösterirken, CCD-18Co hücre hattında anti-sitotoksik etki göstermiştir. Çalışmamızda amigdalinin DLD-1 kanser hücrelerinin canlılığını doza bağlı olarak azalttığı ve CCD18-Co normal epitel hücreleri üzerinde sitotoksik etki göstermediği belirlenmiştir. Amigdalinin diğer anti-tümör ilaçlarla kombinasyon halinde kullanımının fizibilitesini göstermek, bu ilaçların anti-tümör aktivitelerini artırmak ve amigdalindeki aktif bileşenlerin yapay sentezini geliştirmek için daha kapsamlı kontrollü klinik çalışmalara ihtiyaç vardır.

Anahtar sözcükler: Amigdalin, Kanser tedavisi, Hücre kültürü, Kolon kanseri, Vitamin B17

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Introduction

Colorectal cancer is one of the most common cancers in both sexes. Although surgical treatment, radiotherapy, and chemotherapy applications have increased good prognosis rates, approximately 5.8% of the population over the age of 50 are diagnosed with colon cancer, and most of these patients die due to a tumor [1]. Alcohol, obesity, smoking, a sedentary lifestyle, a diet rich in fat, and a diet without fiber are among the important risk factors for colon cancer [2,3]. Some studies in recent years have shown that many natural products are effective against cancer cells [4,5]. Amygdalin is one of them [6,7]. Amygdalin (D-mandelonitrile-β-D-gentiobioside; vitamin B17; formerly laetrile) is found in plant seeds such as apricot, peach, plum, bitter almond, apple, pear, and cherry, and is in the aromatic cyanogenic glycoside group [8]. Amygdalin consists of two glucose molecules, hydrocyanic acid, and a benzaldehyde group. While hydrocyanic acid has anti-tumor properties in its components, the benzaldehyde group has analgesic properties [9]. Some studies have reported that amygdalin has an anti-tussive, anti-atherosclerotic plaque, ulcer-suppressing, anti-inflammatory, and tumor-suppressive effects [10-13]. *In vitro*, amygdalin has been shown to be effective against malignant tumors such as breast cancer [14], prostate cancer [15], colon cancer [16-19], bladder cancer [20], and leukemia [21]. It is accepted that one of the most important mechanisms in the toxic effect of amygdalin against such malignant cells is its stimulation of apoptosis, which is programmed cell death, via caspase-3 and BAX protein [15,22]. Due to the limited number of studies investigating the relationship between amygdalin and colon cancer in the literature, this study aimed to evaluate the cytotoxic and anticytotoxic effects of amygdalin in human colon cancer cells (DLD-1) and normal colon epithelium (CCD-18Co) using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test.

Materials and Methods

DLD-1 colon cancer cells (ATTC®HTB-38™) and CCD-18Co normal colonic epithelial cells (ATCC® CRL-1459TM) were used in our study. Cells were grown in a humid environment at 37°C in a 5% CO_2 incubator. DLD-1 and CCD-18Co cells were grown in flasks containing Roswell Park Memorial Institute-1640 (RPMI-1640) and Eagle's Minimum Essential Medium (EMEM), respectively. These media additionally contain 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells that developed and reached sufficient numbers were inoculated into 96-well plates at 10^4 cells per well. The cells attached to the plate surface were treated with different concentrations of amygdalin. A 100 mM master stock was prepared by dissolving amygdalin (Cayman Chemical, Lot: 364-9897, USA) in dimethyl sulfoxide (DMSO). Concentrations of 100, 50, 25, 12.5, 6.25, 3.125, and 1.56 mM were obtained by dilutions from this master stock (DMSO, Sigma, USA). Both cell groups were treated with these concentrations of amygdalin for 24 hours. The DMSO ratio did not exceed 1% in all the applied concentrations. One negative, one positive control, and DMSO control were also included in the study. All studies were performed in triplicate. When the treatment time with amygdalin was finished, 20% MTT dye was added to the wells of the aspirated plates and incubated for 3 hours. After the reaction was stopped with pure DMSO at the end of the period, the absorbance values of the plates were read spectrophotometrically (Thermo Fisher Scientific/Multiscan) at a wavelength of 570 nm. The applied concentrations and the % cell viability curve were determined using Microsoft Excel (2016 version, Microsoft Corporation, USA) software. The 50% inhibitory concentration value (IC50) was then calculated by utilizing a bar graph. The following formulas were used in the calculations:

- Viability (%) = Average experimental (optical density) OD value / Average control OD value x 100%
- Cytotoxicity = Test absorbance value / Control absorbance value average x100

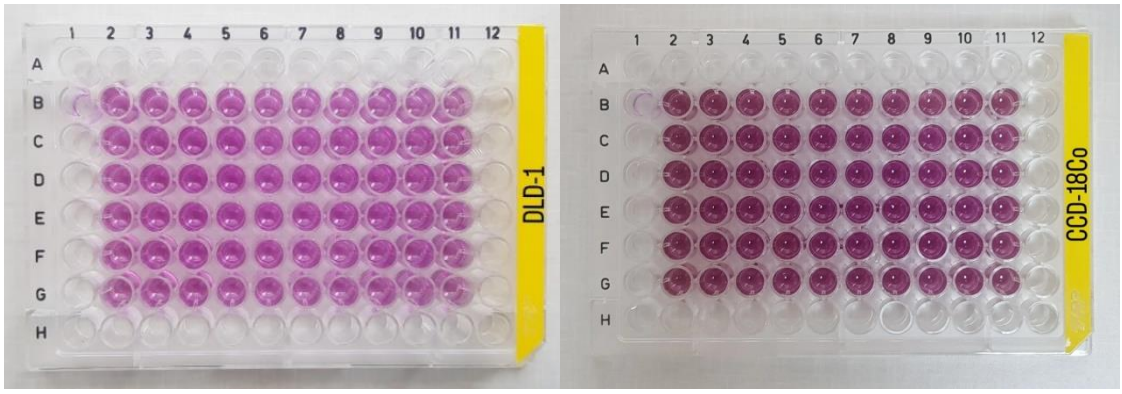

Figure 1. Images of DLD-1 and CCD-18Co cells in the plate

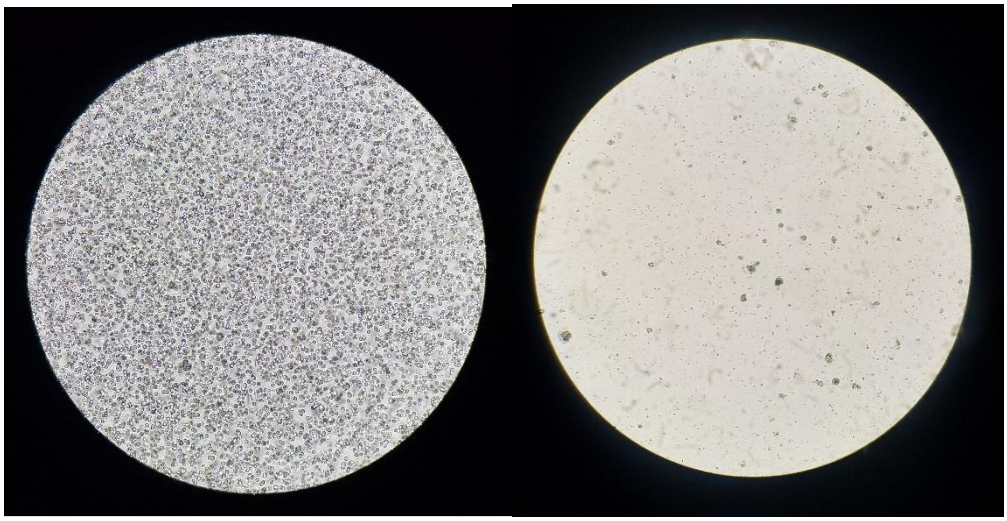

Figure 2. Microscopic images (10X) of DLD-1 and CCD-18Co cells, respectively.

In Figure 1 and Figure 2, images of the plates and cancer cells are given.

According to the obtained data, the cytotoxic effects of amygdalin on cell lines were evaluated and the data were compared with the negative control and positive control groups.

Results

Amygdalin induced concentration-dependent cytotoxicity in the DLD-1 cell line. According to the results obtained, the percent viability values for the DLD-1 cell line were found to be between 48.3-71.6%. The IC50 value for the DLD-1 cell line was calculated as 74.03 mM. The calculated values are given in Table 1 and Figure 3.

Table 1. % Viability and absorbance values of amygdalin in DLD-1 cell line

	Absorbance	% Viability values
Cell Control	2,8792	100
Mitomycin-C	1,6230	56,3698
100 mM	1,3908	48,3050
50 mM	2,0220	70,2278
25 mM	2,0625	71,6344
12,5 mM	1,9226	66,7754
6,25 mM	2,0637	71,6761
3,125 mM	1,9117	66,3969
1,5625 mM	1,7772	61,7254
DMSO	2,5037	86,9581

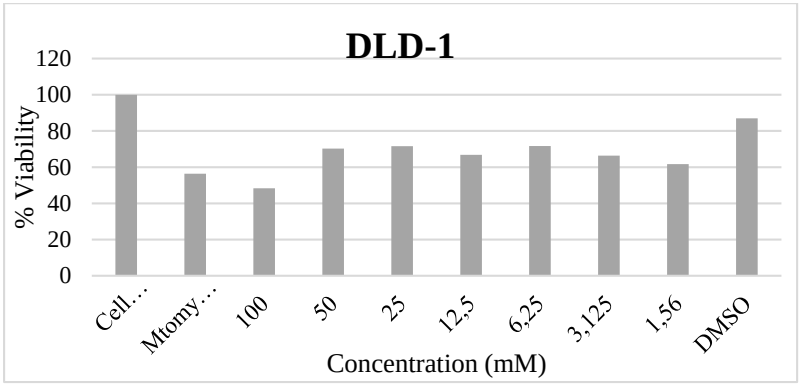

Figure 3. The comparison of viability values of amygdalin in DLD-1 cell line according to concentrations

The viability values for the CCD-18Co cell line after amygdalin treatment ranged from 101.6 to 117.9%. While amygdalin showed a cytotoxic effect in the DLD-1 cell line, it showed an anticytotoxic effect in the CCD-18Co cell line (Table 2, Fig.4).

Table 2. % The viability and absorbance values of amygdalin in the CCD-18Co cell line

	Absorbance	% Viability values
Cell Control	2,3919	100
Mitomycin-C	1,5397	64,3714
100 mM	2,5111	104,9835
50 mM	2,6521	110,8784
25 mM	2,4304	101,6096
12,5 mM	2,3929	100,0418
6,25 mM	2,5045	104,7076
3,125 mM	2,5971	108,5790
1,5625 mM	2,8210	117,9397
DMSO	2,0766	86,81801

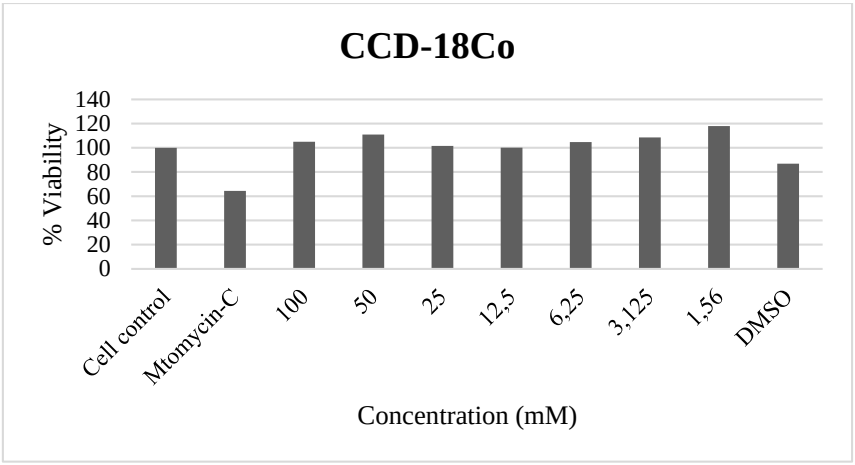

Figure 4. The comparison of viability values of amygdalin in CCD-18Co cell line according to concentrations

Discussion

Many studies have been conducted to improve the prevention and prognosis of colon cancer, whose incidence is increasing worldwide [23-25]. Our study is one of the limited numbers of studies investigating the relationship between colon cancer and amygdalin. With the MTT test, the number of viable cells can be reliably determined. This test has a very important

place in the evaluation of the cytotoxicity of anti-cancer drugs. In our study, using the MTT test, it was determined that amygdalin decreased the viability of DLD-1 cancer cells in a dose-dependent manner and did not show cytotoxic effects on CCD18-Co normal epithelial cells. In addition, the IC50 values obtained after our experiments may be useful in determining the starting dose in *in vivo* studies with experimental animals such as mice, thus reducing the number of animals needed significantly. We suggest that amygdalin could be developed as supportive therapy for colon cancer.

In recent years, some studies, though very few, have been published investigating the effects of amygdalin on colon cancer cells. Park et al. reported that amygdalin inhibited the proliferation of SNU-C4 colon cancer cells by suppressing the expression of cell cycle genes (ATP-binding cassette, exonuclease 1, topoisomerase I, and sub-family F) [16]. RT-PCR analyses revealed that the mRNA levels of these cycle genes in the SNU-C4 cell line were reduced by amygdalin treatment. The common aspect of this study with our study was that the MTT test was applied. However, we did not apply the RT-PCR and cDNA microarrays used in that study. The results of the above study by Park et al. and the results obtained from our research support each other.

In a study conducted on albino rats with colon cancer in Egypt in 2019, the effect of vitamin B17 was investigated [17]. Rats were divided into treatment and control groups. Tissue samples were taken from the groups for microscopic examinations and morphological analyses were performed. Morphological findings in favor of malignancy (pleomorphism, hyperchromatism, dysplasia) were found to be significantly higher in the cancer group and treated group ($p<0.0001$). Being an *in vivo* study was an important advantage compared to our study.

A study investigating the relationship of amygdalin with colorectal carcinoma (HT-29) and hepatocellular carcinoma (HepG2) cell lines was published by Dimitrov et al. in 2021 [18]. In this study, amygdalin concentrations were determined by reverse-phase HPLC. Furthermore, antigenotoxic, antimutagenic, and anticarcinogenic effects of amygdalin were reported. HepG2 was the most sensitive group among the cell lines studied. The most important positive aspects of this study compared to ours were that the research in that particular study was conducted with different cancer cell lines and the amygdalin concentration was measured with a very high accuracy method.

Other than colon cancer, various articles investigating the relationship between many cancers and amygdalin have been published. It has been reported that amygdalin treatment increases cellular death via apoptosis in prostate [15], bladder [20], leukemic [21], breast [22], liver [19,22], lung [22], and cervical cancer cells [26].

Conclusion

More comprehensive controlled clinical trials are needed to demonstrate the feasibility of using amygdalin in combination with other anti-tumor drugs and to develop the artificial synthesis of the active ingredients in amygdalin in order to increase the anti-tumor activities of these drugs. Cyanide in amygdalin is considered to have an anti-cancer role. After cancer cells break down amygdalin, cyanide is released and kills the malignant tumor cell. It is thought that the enzyme rhodanese, which inhibits the toxic effect of cyanide, is found in lesser quantities in cancer cells. This enzyme converts cyanide to the less harmful thiocyanate. This may explain why cancer cells are more sensitive to amygdalin. The β-glucosidase enzyme is thought to be higher in malignant tumor cells. This enzyme is a protein that can degrade amygdalin and cause toxicity in cancer cells [27].

Considering the negative aspects of the treatment process of patients with malignant tumors and the negative effects of the treatment protocols using chemical drugs, the planning and researching the use of amygdalin, which has antioxidant and anticarcinogenic effects and supports health, is of great importance for the future. In our study, we think that amygdalin, which we have seen *in vitro* during cancer treatment, may be effective in repairing DNA damage and preventing the side effects of some drugs. Due to the increase in the use of food supplements among cancer patients, we think that our study will make an important contribution in terms of revealing the effects of recently popular research topics such as amygdalin on tumor cells more clearly and also will help in raising the awareness of healthcare professionals on this issue.

Study limitations: The most important limitations of our study were that the study was not supported by PCR, Real-time PCR, and Western Blot, and also gene expressions were not examined.

Another limitation was that we only worked with a single cancer cell type. Despite all this, we plan to add these methods in our future studies for examining different possibilities and to investigate the effect of amygdalin on various cancer cells.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethical approval: Ethics committee approval was not obtained by the authors as there were no human participants and this was a cell culture study.

Author Contributions: AO conceptualized the main idea and hypothesis of the study, developed the theory, arranged the material and method section, evaluated the data in the results section, wrote the discussion section, reviewed, and made the necessary arrangements, and approved the manuscript. AAK, BK, and EAY conceptualized the main idea and hypothesis of the study, evaluated the data in the results section, wrote the discussion section, reviewed, and made the necessary arrangements, and approved the

manuscript. The authors have read and approved the final manuscript.

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