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Investigation of Ellagic Acid in Human Colon Cancer Cells

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Abstract

Cancer is seen as one of the most important and deadly diseases of our time. Colon cancer, which is one of the cancer types and ranks second in cancer-related deaths, is the second most common cancer type in men and the fourth most common cancer type in women. Ellagic acid is a plant phytochemical that can be obtained from fruits and vegetables such as strawberries, raspberries, and hazelnuts and is very important for human health. In order to understand the effects of ellagic acid on human colon cancer, different doses of ellagic acid were applied. In the proliferation study, ellagic acid was applied to CCL-233 cells at concentrations of 5 µM, 10 µM, 25 µM, 50 µM and 100 µM. Cell death was observed with an increase in applied ellagic acid concentration. The semi-lethal dose (LD50) was determined to be 40 µM. For poly (ADP-ribose) polymerase (PARP) and vascular endothelial growth factor (VEGF) studies, different doses of ellagic acid were applied to human colon cancer cells, which consequently showed a decrease in both values.

Keywords: *Ellagic acid, cell culture, MTT, PARP, VEGF.*

Elajik Asidin İnsan Kolon Kanseri Hücrelerinde İncelenmesi

Özet

Kanser çağımızın en önemli ve en ölümcül hastalıklardan biri olarak görülmektedir. Kanser türlerinden biri olan ve kansere bağlı ölüm vakalarında ikinci sırada yer alan kolon kanseri erkekler bireylerde en yaygın ikinci, kadınlarda ise en yaygın dördüncü kanser tipidir. Elajik asit çilek, ahududu, fındık gibi meyve ve sebzelerden elde edilebilen ve insan sağlığı açısından oldukça önemli bir bitkisel fitokimyasaldır. Elajik asidin insan kolon kanseri üzerindeki etkilerinin anlaşılması için, farklı dozlarda elajik asit uygulanmıştır. Yapılan proliferasyon çalışmasında CCL-233 hücrelerine 5 µM, 10 µM, 25 µM, 50 µM ve 100 µM konsantrasyonlarında elajik asit uygulanmıştır. Uygulanan elajik asit konsantrasyonun artması ile beraber hücrelerin ölümü gözlemlenmiştir. Yarı öldürücü doz (LD50) 40 µM olarak tayin edilmiştir. PARP ve VEGF çalışmaları için insan kolon hücrelerine farklı dozlarda elajik asit uygulanmış ve sonuç olarak iki değerde de azalma görülmüştür.

Anahtar Kelimeler: *Elajik asit, hücre kültürü, MTT, PARP, VEGF.*

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1. Introduction

Cancer is one of the deadliest and most dangerous diseases of our time. Colon cancer, which is one of the types of cancer named according to the region where they occur or develop on the human body, is the name given to the types of cancer seen around the large intestine and rectum in humans. Colon cancer (CRC) is a type of cancer seen in one of the tissues of the colon, which is the longest branch of the intestine, and in tissues of the rectum, which is the majority of the intestine in front of the anus. Colon cancer is a very risky cancer group in terms of both incidence and mortality. It is seen in many individuals worldwide. CRC are usually seen as small nodules or polyp structures in the colon or rectum. In the future, these structures may proliferate uncontrollably and cause cancer formation in the tissues. These polyps, which are generally known as benign, can cause cancer. This process can be observed for an average of 10 years. Colon cancer is the second most common cancer in men and the third most common in women [1].

In terms of colon cancer spread, although it is seen all over the world, it is more common in regions such as North America, Western and Eastern European countries, and Australia, which are more developed economically and technologically. On the other hand, in many Asian countries and especially in African countries, both the spread and the mortality rate due to the disease are very low compared to other countries. These data also show that the food consumed by society and lifestyles have a very important effect on the spread and mortality rates of CRC [2].

The consumption rate of phenolic chemicals in the diet is seen as a factor affecting the health of individuals. It is thought that such chemicals are generally taken from fruit and vegetable species. It is seen that the rates of catching many diseases, such as cancer, cardiovascular diseases, and diabetes, are lower in societies with such diets [4][5].

Ellagic acid is a phenolic lactone compound that can be found naturally in various plant species, especially fruits. The chemical formula of ellagic acid is $C_{14}H_6O_8$. Its molecular weight is 302.197 g/mol, and its density is 1.67 g/m^3. There is a benzene ring in the polyphenol structure [6]. In these plant species, chemicals called ellagitannin, which are seen as cellular structure elements, are present. They can be easily obtained by hydrolysis. They can be seen in high concentrations in many fruits. Ellagic acid, which is especially common in strawberries, raspberries, cranberries, and grapes, is also found in shelled foods such as walnuts and hazelnuts [7][8].

Studies have shown that ellagic acid is a strong anticarcinogen. The acid is involved in the removal of toxin groups in the tumor area or damaged tissue. In this way, it is very important in terms of preventing the onset of carcinogenesis in the region [9].

In this study, the effects of ellagic acid on human colon cancer cells (CCL-233) and its suitability for use in a new treatment method were evaluated.

2. Materials and Methods

CCL-233 cells, which are human colon adenocarcinoma cells, were used in the study. The cells used were provided by Celal Bayar University's Faculty of Medicine, Histology, and Embryology Laboratory.

2.1. Cell Culture

Cell culture studies were carried out in the Uşak University Scientific Analysis and Technological Application and Research Center Cell Culture Research Laboratory. The cancer cell line used in the experiments was obtained from Manisa Celal Bayar University. Cancer cell lines arrived in 25 cm^2 flasks. The cell line used is CCL-233 SW-1116 human colon cancer cells. The medium used for CCL-233 SW-1116 cells was 10% RPMI-1640 (with L-glutamine), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Using this medium, the cells were taken into 25 cm^2 and 75 cm^2 flasks, and they were kept in incubators that provided 5% CO_2 and 37°C conditions for their growth media. After cell growth, cell passages were performed in a laminar flow cabinet. An inverted microscope was used to examine cell cultures.

2.2. Cell Passage

Cells were grown in flasks containing 4 ml of 25 cm^2 flasks and 12 ml of RPMI-1640 (with L-glutamine) medium containing 10% FBS and 1% penicillin/streptomycin in 75 cm^2 flasks. When the cell density reached the 80–90% band, the cells were re-passaged and replicated. Before cell passage, cells must reach a certain density in flasks. In our study, cells were allowed to grow until the cell densities in the flasks were 80–90%. The medium was withdrawn from the flasks of the cells that reached this density. After this stage, phosphate buffered saline (PBS) was added as 8 ml for 25 cm^2 flasks and 24 ml for 75 cm^2 flasks. The removal of dead cells in the flask was accomplished by removing PBS. Since colon cancer cells are a sticky cell group, 0.25% trypsin was added in amounts of 2–2.5 ml to 25 cm^2 flasks and 5-7 ml to 75 cm^2 flasks to separate them from the flasks. After adding trypsin, the cells were kept in a 37 °C incubator for a while. In order to remove the effect of trypsin on the cells, an average of two times the amount of trypsin was added, and the cells were transferred to centrifuge tubes with the help of a sterile pipette. The cells were centrifuged at 1500 rpm for 5 minutes to remove the supernatant. The centrifuged cells were homogenized by adding 2 ml of medium for 25 cm^2 flasks and 4 ml for 75 cm^2 flasks, and the medium was added to them and preserved in flasks. Cells were removed to a 37 °C incubator until the next passage.

2.3. Cytotoxicity and Proliferation

By applying different concentrations of ellagic acid to the human colon cancer cell line CCL-233 cells, the effect of ellagic acid on cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. CCL-233 cells were seeded in 96-well plates, with cells in 3 replicates for each dose of ellagic acid to be applied. They were cultured for 24 hours in an incubator at 37°C and 5% CO_2. At the end of 24 hours, ellagic acid prepared in dilution (5 µM, 10 µM, 25 µM, 50 µM and 100 µM) was given to the cells at different concentrations. Cells were cultured for 48 hours at 37°C in a 5% CO_2 incubator. At the end of the period, the medium of the cells was collected, and the wells were washed with PBS. The cells were given fresh medium containing 10% MTT reagent and incubated for 3 hours in the incubator. At the end of the incubation, 20% MTT solvent was applied to each well. The samples, which were kept at room temperature for 15 minutes, were read with a microplate reader spectrophotometer (µ-Quant, BioTek Instruments, Winooski, Vermont, USA) at a wavelength of 570 nm. The semi-lethal dose (LD50) of ellagic acid on CCL-233 human colon cancer cells was determined with the obtained data.

2.4. PARP

Cells for PARP analysis were taken from 75 cm^2 flasks. Cells were removed from the plates in a similar manner as passages. Then it was taken into centrifuge tubes for centrifugation. After centrifugation, the cells were suspended by adding medium to the tube. 24-well plates were used for this analysis. Average $5x10^5$ CCL-233 SW-1116 cells were seeded into the wells at 450 µl. Cells were removed from the incubator for 24 hours. After incubation, different doses of ellagic acid were added to the wells. Cells were removed to the incubator for 48 hours after acid addition. After the incubation period, the media on the plates were withdrawn with the help of a pipette. After that, 700 µl of PBS was added to the plates. After a while, PBS was aspirated with a pipette, and 200 µl of trypsin was added to the wells to change the adherent status of the cells. In order for the effects of trypsin to take place, 450 µl of medium was added to the cells that were left in the incubator for a certain period of time to prevent further damage. Cells were taken into epondorfs with disposable pipettes. Cells in Eppendorf tubes were centrifuged at 2100 rpm for 20 minutes at 15°C. The supernatant that formed after this procedure was removed. The medium was added to the Eppendorfs. Using the antibodies from the PARP kit, 96-well plates were inoculated. Three different groups were formed: reagents, samples, and standards. The cells were cultivated and transferred to an incubator at 37°C for 60 minutes. After incubation, the plates were washed five times. After washing the cells, chromogen solutions A and B were added and incubated at 37°C for 10 minutes. At the end of the time, stop solution was added, and measurements were made with the Plate Reader.

2.5. VEGF

Cells for VEGF analysis were taken from 75 cm^2 flasks. Cells were removed from the plates in a similar manner as passages. Then it was taken into centrifuge tubes for centrifugation. After centrifugation, the cells were suspended by adding medium to the tube. 24-well plates were used for this analysis. Average $5x10^5$ CCL-233 SW-1116 cells were seeded into the wells at 450 µl. Cells were removed from the incubator for 24 hours. After incubation, different doses of ellagic acid were added to the wells. Cells were removed to the incubator for 48 hours after acid addition. After the incubation period, the media on the plates were withdrawn with the help of a pipette. After that, 700 µl of PBS was added to the plates. After a while, PBS was aspirated with a pipette, and 200 µl of trypsin was added to the wells to change the adherent status of the cells. In order for the effects of trypsin to take place, 450 µl of medium was added to the cells that were left in the incubator for a certain period of time to prevent further damage. Cells were taken into Eppendorf tubes with disposable pipettes. Cells in Eppendorf tubes were centrifuged at 2100 rpm for 20 minutes at 15°C. The supernatant that formed after this procedure was removed. The medium was added to the Eppendorf tubes. Using the antibodies from the VEGF kit, 96-well plates were inoculated. Three different groups were formed: reagents, samples, and standards. The cells were cultivated and transferred to an incubator at 37°C for 60 minutes. After incubation, the plates were washed five times. After washing the cells, chromogen solutions A and B were added and incubated at 37°C for 10 minutes. At the end of the time, stop solution was added, and measurements were made with the Plate Reader.

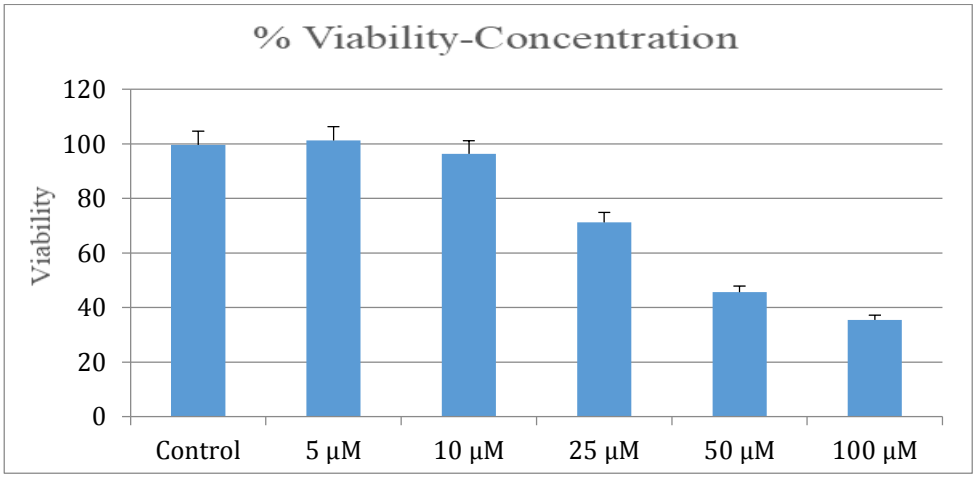
2.6. Statistical Analysis

In our study, ELISA device test results were analyzed using the SPSS-20 statistical program. Results are given as mean (SE) standard error (SE = SD/n). After testing the homogeneity of the groups, Duncan was used in the one-way ANOVA test to find the differences between the groups. Differences were considered statistically significant at $P < 0.05$.

3. Results

The results of the proliferation study are shown in Table 1.

Table 1. Proliferation test results



The CCL-233 cell line, which is made up of human colon cancer cells, was used in the proliferation and cytotoxicity experiments. In our study, the MTT kit was used to understand the effects of ellagic acid. The concentration ranges of the cells were determined as 5, 10, 25, 50, and 100 µM. The viability rates of the cells were examined by applying the MTT kit to the cells after incubation for 48 hours. The dose that caused half of the cells to die (LD50) was calculated as 40 µM. According to the MTT test results obtained, it was observed that ellagic acid had a lethal effect on human colon cancer CCL-233 cells, depending on the increase in the dose.

Table 2. PARP and VEGF analysis results

	PARP (ng/L)	VEGF (ng/L)
Control	2,3756±0,07[a]	0,0310±0,0075[a]
Ellagic acid 20 µM	1,7930±0,05[b]	0,0290±0,0050[a]
Ellagic acid 40 µM	1,5100±0,03[b]	0,0261±0,0443[a]
Ellagic acid 80 µM	1,3484±0,02[b]	0,0217±0,0200[a]

a, b, c: Same letters in the same column are not significantly different according to the ANOVA-Duncan test ($P<0.05$).

Poly-ADP-ribose-polymerase (PARP) and vascular endothelial growth factor (VEGF) tests were applied to better understand the effects of ellagic acid on human colon cancer cells. 20, 40, and 80 µM ellagic acid were applied to the cells, and the control group was also included in the test. Looking at the results of the tests, it is thought that ellagic acid may help cell apoptosis and slow down angiogenesis. The results are shown in Table 2.

4. Discussion

Colon cancer is a type of cancer that spreads worldwide and is increasing due to many reasons. When we look at external factors, colon cancer, which increases day by day due to factors such as diet, alcohol consumption, smoking, and harmful substance use, can also be seen in humans due to genetic reasons. Colon cancer, which is generally seen due to external factors, is seen as a very deadly type of cancer with a high mortality rate, except for individuals who have a higher risk of catching the disease caused by genes that can come from within the family. When we look at the treatment methods applied for colon cancer, it is seen that methods such as surgery, radiotherapy, and chemotherapy are used, although they vary depending on the stage. It is known that methods related to the use of chemical drugs damage healthy cells as well as cancerous cells. In addition to chemical drug treatments that are still being developed and researched, there are applications such as biotherapy and immunotherapy that are less damaging to healthy cells [10].

Ellagic acid is a phenolic phytochemical. Ellagic acid, which is abundant in fruits such as pomegranates, raspberries, strawberries, and nuts such as hazelnuts and walnuts, is a very important substance in terms of protecting human health and fighting against diseases. As a result of years of research on ellagic acid, it has been revealed that it can be used against cancerous cells. It has been observed that it helps to remove toxins, especially from the cancerous structure and damaged cells in its region, thus preventing the initiation of carcinogenesis in the region [9].

The aim of our study is to develop an alternative treatment method and understand the effects of ellagic acid, which we think can be used in this field. In another study, different doses of ellagic acid were applied to HCT-116 and HC-29 cells, which are human colon cancer cells. The MTT test was applied to the cells treated with 10–50 μM doses of ellagic acid, and the viability rates of the cells were examined. It was observed that the death rate in the cells increased depending on the increase in the dose. As a result of the application of a 10 μM ellagic dose, the viability rate in HCT-166 cells decreased to 86.8%, while this rate decreased to 24.1% after 50 μM. Likewise, 90.2% viability was observed in HC-29 cells at a dose of 10 μM, while a viability rate of 30.1% was obtained at a dose of 50 μM [11]. In our study, in CCL-233 cells, which is a different human colon cancer cell line, we saw that the viability of the cells decreased due to the increase in the dose of ellagic acid. As a result of the studies, it is seen as a sufficient result to say that ellagic acid has an antiproliferative effect on cells.

In an in vitro study, they infected PANC-1 cells, which are human pancreatic cancer cells, into Balb/c-type mice and observed the formation of cancer cells in the pancreas of the mice. After the cancerous tissues reached a certain size, ellagic acid was applied to the mice, and the results were observed. In light of the data obtained, it was determined that the carcinoma structures in the pancreatic regions of the mice shrank and decreased in volume as a result of the measurement tests. It has also been discovered that it prevents the further spread of cancer. It was aimed at understanding the effects of ellagic acid on other organs by looking at tissues other than the target organ, and no cytotoxicity was observed in the liver, spleen, and lung tissues of mice [12]. This study has proven that ellagic acid is effectively used not only in in vivo but also in vitro studies. It is also thought that ellagic acid prevents the spread of cancer and is a treatment method that can be applied to reduce cancer structures in tissues.

In another study, ellagic acid was studied on human prostate cells (PC-3 and PSL-10 cells). The ellagic acid obtained from the pomegranate fruit has been tested on both humans and mice with cancer. Considering the test results obtained, it was seen that ellagic acid greatly reduced the spread of cancerous tissues in cancer mice and caused the death of cells. In addition, as a result of cell culture studies, it was observed that cells dragged themselves into apoptosis and their spreading rate decreased [13]. When the results of this study are examined, it is thought that ellagic acid, which almost does not harm the surrounding healthy cells, and can be used as a treatment, should be used as a different method than common treatment methods.

In our study, the lethal effects of ellagic acid on CCL-233 cells, which are human colon carcinoma cells, were investigated by the MTT test. Within the scope of the study, five different doses of ellagic acid were applied. Depending on the increase in the dose of ellagic acid applied to the CCL-233 cells, an increase in the death rate of the cells was observed. In the applied doses of ellagic acid, a toxic effect began to be seen in the cells starting from the 25 µM dose. The LD50 dose that caused half of the population to die was found to be 40 µM [Table 1]. Within the scope of the study, it was observed that the most effective dose was 40 µM in 48 hours of application to the cells treated with ellagic acid. There may be differences in the data obtained as a result of changing the dose of ellagic acid applied or changing the time period to be treated for further studies based on this study. Increasing the amount of ellagic acid to be applied causes an increase in the lethal effect on the cells. For a better understanding of the application, the application of ellagic acid at different doses at longer or shorter time intervals compared to our study is also very important for the data to be obtained. Based on the data we obtained from our study, we think that ellagic acid is a phenolic phytochemical that can be used in cancer treatments. In light of these data, we think that in-vivo and in-vitro studies on ellagic acid can provide a different perspective on cancer treatments.

PARP is a group of enzymes that can be found in the cell nucleus. In particular, it has important effects on the repair of DNA damage. It is seen as an important marker because of its detection and repair of damage to DNA, its involvement in changes to chromatin, and its involvement in necrosis and apoptosis processes in the cell [14]. In our study, ellagic acid applied to human colon cancer cells at concentrations of 20, 40, and 80 µM caused a response that could be seen as a decrease in PARP values [Table 2]. As a result of these decreasing amounts, we think that PARPs cause cellular death rather than repairing DNA. In this way, it is possible to say that the application of ellagic acid helps the death of cancerous cells. Further studies on the subject are important to better understand the effects of PARPs and to analyze how ellagic acid has an effect on PARPs. Especially with a study on PARP breaks, both the working principles of the mechanism and the effects of ellagic acid can be examined in more detail.

Cancer cells want to grow regularly and unlimitedly. One mechanism that is effective during this growth is the vascularization mechanism called VEGF. Cancer cells make vascularization with the VEGF factor they secrete in order to grow, which allows the cells to grow faster. Therefore, VEGF rates increase with the increase of cancerous cells, and VEGF rates decrease in cases of resistance [15]. In our study, VEGF values decreased after ellagic acid was applied to human colon cancer cells at 20, 40, and 80 µM concentrations [Table 2]. Considering these values, it can be said that ellagic acid prevents the vascularization of cancerous cells. The use of ellagic acid in different doses in different cell types in order to better understand the effects of angiogenesis can be considered a study to explore the full effect potential of the substance.

Jaman and Sayeed conducted in vitro studies to understand the effects of ellagic acid on breast cancer. As a result of studies on different breast cancer cell lines, they have seen that ellagic acid reduces proliferation. Similarly, similar results were obtained in our study. However, they observed that ellagic acid decreased the expression of PARP, and as a result, the cells underwent apoptosis. When we look at the data we have obtained as a result of our studies, it has been observed that the PARP values have decreased [16].

Ceci et al. used human bladder cancer cells in their studies. Ellagic acid was applied to four different cancer lines, and the effects were observed. Similar to the results of our study, reductions were detected as a result of the proliferation test. As seen in many studies, death is observed at different rates depending on the increase in the dose of ellagic acid. VEGF-A and VEGF-2 concentrations were observed in the study, and a decrease was observed depending on the dose increase. It was thought that as a result of the decrease in VEGF values, angiogenesis slowed down and a decrease in the spread rate of cancer cells occurred. In our study, there was a significant dose-dependent decrease in VEGF values [17].

Matin et al. investigated the effects of ellagic acid on human gastric cancer cells. As the side effects of many treatment methods cause great destruction in the body, ellagic acid has been considered a different treatment method. In the study, different doses of ellagic acid were applied to human gastric cancer cells, and the death of the cells was observed depending on the dose. In addition, in animal experiments, human gastric cancer cells were injected into mice, and changes were observed by applying ellagic acid. As a result, the death of tumor structures in mice was observed depending on the dose given, and it was revealed that ellagic acid has an anti-proliferative effect, as in our study [18].

Ni et al. investigated the apoptotic and autophagic effects of ellagic acid on human colon cancer cells. In the study, different doses of ellagic acid were applied to human colon cancer cells. When the results were examined, it was observed that the death rate of the cells increased despite the increase in the dose. Similar results were observed in our study as well. In genetic studies, it has been seen that ellagic acid affects gene groups that are effective in cell apoptosis and has an apoptosis-increasing effect. It has been demonstrated that ellagic acid, which has been shown to accelerate both apoptosis and autophagy through AMPK and mTOR pathways, can be used as a different treatment method against colon cancer [19].

In the study of Vanella et al., different doses of ellagic acid were applied to human prostate cancer cells. The study investigated how ellagic acid affects certain factors that help cancer grow. VEGF, one of these factors, decreased in a dose-dependent manner. Similar results were obtained in our study. In addition to this factor, a decrease was observed in the levels of different stimuli, such as fibroblast growth factor, granulocyte colony stimulating factor, and hepatocyte growth factor, with increasing dose. In the light of these results, it has been revealed that ellagic acid has a negative effect on human prostate cancer cells and is a chemical that can be used to slow down the growth and spread of cancer cells [20].

In another study, Wang et al. examined ellagic acid using both in vitro and in vivo studies to understand its effects on breast cancer. It has been observed that the VEGF-2 factor, which is thought to affect the growth rate of cancerous cells and is related to the rate of spread, decreases with the prolongation of the waiting time, together with the applied ellagic acid doses. Similar to our study, in this study, which included different doses and different application times, a decrease in the VEGF factor was observed with an increase in the applied dose and the duration of application. In the light of these results, which are

thought to affect angiogenesis negatively, it is thought that the chemical can be used as a different treatment method [21].

Our study has proven that ellagic acid also has a negative effect on cancer cells in human colon cells (CCL-233). It was observed that the lethal effects of ellagic acid, as a result of the MTT test, had a significant effect on the averages with PARP and VEGF analyses. We think that more comprehensive and detailed studies on the subject may be an alternative method to the conventional treatment methods of ellagic acid. In this way, the currently more widely used treatment methods are not only healthy for cancer cells; the damage to the body can be prevented, and it can be predicted that sick individuals will return to a healthier life with less damage from their side effects. In addition to cancer treatment, it can be used for the consumption of foods containing ellagic acid for daily survival.

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Conflict of Interest

There is no conflict of interest in this study.

Ethics Consent

Ethical consent is not required for the study.

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