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Araştırma Makalesi - Research Article

Ellajik Asidin İnsan Akciğer Kanseri Üzerine Antiproliferatif Etkinliklerinin İn Vitro Araştırılması

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ÖZ

Akciğer kanseri dünyada yüksek ölüm oranlarına sahip olan bir kanser türüdür. Bitkilerde doğal olarak bulunan polifenollerin antioksidan özellikleri bilinmektedir. Polifenoller biyolojik aktiviteleri, besinsel ve antioksidan değerleri sebebiyle araştırmalarda yer almıştır. Ellajik asit çilek, kızılcık, ceviz, nar ve ahududu gibi birçok meyvede bulunan doğal bir bileşiktir. Ellajik asit antioksidan özellikleri ile bilinen ve insan vücudunu zararlı serbest radikallerden koruyan bir bileşiktir. Bu çalışmada ellajik asit birçok kanser hücresi üzerinde hücre büyümesini baskılama özelliğinden dolayı akciğer kanseri A549 hücrelerinde hücre ölümünü tetiklemek amacıyla kullanılmıştır. A549 hücrelerine ellajik asidin sitotoksik etkilerini araştırmak için MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide) tekniği kullanılmıştır. Buna ilaveten ince yapısal ve morfolojik değişiklikler TEM ve konfokal mikroskopi teknikleri kullanılarak değerlendirilmiştir. Sitotoksisite test sonuçları uygulama dozunun artması ile hücre canlılığının düştüğünü göstermiştir ve morfolojik ve ince yapısal analizler bu ajanın sitotoksisitesini kromatin yoğunlaşması, membran tomurcuklanması, krista kaybı gibi apoptotik gösterge olarak değerlendirilen değişikliklere yol açarak gerçekleştirdiğini göstermiştir. Sonuçlarımıza dayanılarak ellajik asit farklı kanser hücrelerinde kanser tedavisinde alternatif bir tedavi ajanı geliştirmek amacıyla daha ileri araştırmalar için önerilmektedir.

Anahtar Kelimeler- A549, ellajik asit, akciğer kanseri, TEM, konfokal.

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In Vitro Assessment of Anti-Proliferative Activities of Ellagic Acid on Human Lung Cancer

ABSTRACT

Lung cancer is a cancer type with high mortality rates in the world. Polyphenols found naturally in plants are known for their antioxidant properties. Polyphenols are studied due to their biological activity and nutritional antioxidant potential. Ellagic acid is a natural compound contained in many plants like strawberry, cranberry, walnut, pomegranate and raspberry. Ellagic acid is known for its antioxidant properties and protects the body from harmful free radicals. In this study, due to its potential to suppress cell growth in many cancer cells, ellagic acid, was used to stimulate cell death in lung cancer cells, A549. Cytotoxic effects of ellagic acid on A549 cells were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide) technique. Moreover, ultrastructural and morphological changes were evaluated with TEM and confocal microscopy techniques. Cytotoxicity test results showed that the percentages of viability decreased by an increase in the applied dose and morphological and ultrastructural analyzes showed that the cytotoxicity of this agent caused changes as chromatin condensation, membrane blebbing, loss of cristae that are considered as clear apoptosis signes. Based on our results ellagic acid may be recommended for further research on different types of cancer in order to find an alternative agent for cancer treatment.

Keywords- A549, ellagic acid, lung cancer, TEM, confocal.

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I. INTRODUCTION

Lung cancer is the most prevalent and mortal cancer in the world. The percentage of 85 of lung cancer incidence belongs to non-small cell lung cancer type (NSCLC). Therefore, it is crucial to develop reliable and efficient curing approach and therapeutics to treat this cancer type in order to suggest life prolonging and pain ameliorative option to the sufferers [1]. Recently, there has been an increase in the use of plant-based compounds in new developments for prevention or therapy of various cancers due to their antioxidant roles. Flavonoids and phenolic acids of plant compounds are reported to play a vital role in prevention and treatment of many human diseases [2]. Phenolic compounds belong to plant derivatives comprising an aromatic ring with functional groups and hydroxyl groups in their chemical structure. Lignans, phenolic acids, stilbenes and flavonoids are most experimented groups of compounds. Of these, phenolic acids and flavonoids are important as antioxidants [3].

Ellagic acid is described as a phenolic compound derived from leaves, fruits and seeds of various plants such as jaboticaba, walnut, myrobalan, great burnet, pomegranate. Ellagic acid can be formed by hydrolysis of secondary metabolites of plants-ellagitanins. Antiinflammatory and antioxidant properties of ellagic acid are known in many cancer cells. Futhermore, many studies have shown that ellagic acid extracts of plants have a growth-suppressing effect on cells of different cancer types [4].

Recent studies on cancer research reported the antitumor, antiproliferative, cell cycle suppressing, proapoptotic, anti-metastatic and anti-angiogenic activities on ellagic acid on a variety of cancer cell lines. Despite these biological activities of ellagic acid on human non-small cell lung cancer (A549) has not been known in details yet and remain unclear [5]. Thus, herein it is aimed to investigate the cytotoxic and anti-proliferative activities of ellagic acid on human lung cancer *in vitro*, together with the possible ellagic acid-derived alterations in the cell structure.

II. EXPERIMENTAL STUDY

A. *Materials*

A549 (ATCC® CCL-185™) cells were obtained from the American Type Culture Collection (ATCC) (Manassas, USA). Ellagic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide (MTT), fetal bovine serum, penicillin-streptomycin, dimethyl sulfoxide (DMSO) and were purchased from Sigma-Aldrich (St. Louis, USA) and Roswell Park Memorial Institute medium (RPMI-1640) was from GIBCO (Grand Island, USA).

B. *Cell culture and cytotoxicity assay*

A549 cells were cultured in RPMI-1640 medium containing penicillin-streptomycin (100 units/mL-100 µg/mL) and fetal bovine serum (10%) at 5% CO_2. In all experiments, cell culture flasks of at least 85% of confluence were used and experiments were performed in triplicate. For the continuity of cells passage of the cells were performed each third day. For the cytotoxicity MTT assay was realized. For this manner, an ellagic acid stock solution (in DMSO) was prepared and further dilutions were made in fresh complete culture medium (RPMI-1640) until the final DMSO concentration per well did not exceed 0.1-0.2%. Firstly, A549 cells of $5x10^3$ / well were seeded in 96-well plates and concentrations of 3.13-200 µM of ellagic acid were added in the wells. The treated cells were incubated for 24 hours under the same culture conditions. Following the incubation, 20 µL / well of MTT solution (5 mg / mL) was added and allowed to incubation further during 3 hours. At the end of incubation, MTT dye was dissolved by adding DMSO (200 µL / well) and plates were read on plate reader (HTX Synergy, BioTek, USA) at a wavelength of 570 nm (n = 3) [6]. The percentages of viability were calculated relative to the untreated group of cells. The half-maximal inhibitory concentration (IC_{50}) was detected from the calculated viability percentages.

C. *Evaluating the morphological alteration effects of ellagic acid*

Confocal microscopic evaluation was performed to determine the morphological changes of A549 cells exposed to ellagic acid. Firstly, A549 cells cultured on cover slips in six-well culture plates and incubated at 37°C in a cell culture incubator containing ellagic acid at IC_{50} for 24 hours. An untreated group of A549 cells

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were grown at the same conditions and were used as control cells. After incubation, all samples were cleared in phosphate buffered saline (PBS, Invitrogen, USA) and fixed in glutaraldehyde (2%) at room temperature (20 minutes). The fixed cells were stained binary in Alexa fluor-488 phalloidin and acridine orange. All samples were imaged under a Leica ICS-SP5 II confocal microscope supported by adequate software (Leica Confocal Software Version 2.00, Leica, Germany).

D. Structural Analysis of ellagic acid treated cells

To examine the changes in the fine structure of A549 cells under transmission electron microscope (TEM) the cells were grown on cell culture plates (75 cm^2) and treated with IC_{50} concentration of ellagic acid for 24 hours in the same incubator conditions. After the incubation cell samples were fixed in glutaraldehyde (2.5%) and in osmium tetroxide. The fixed cells were dehydrated in grade ethanol and embedded in Epon 812 epoxy. Embedded samples were allowed to polymerization for 48 hours at 65°C. Obtained blocks were sectioned with an ultramicrotome (Leica EMUC6, The Nederland). The thin sections were stained with uranyl acetate and lead citrate, and evaluated under TEM (FEI Tecnai BioTWIN, The Nederland).

E. Statistical Analysis

The statistical analysis of the research data was used one way variance analysis for multiple comparisons of GraphPad Prism 6.0 for Windows.

Conflicts of Interest

Authors do not claim any kind of conflicts of interest for this study.

III. RESULTS

A. MTT Results

The viability inhibition curve (Figure 1) of A549 cells exposed to ellagic acid IC_{50} concentration for 24 hours was prepared with the Microsoft Office Excel program. The IC_{50} value was determined as 30 µM for this application time.

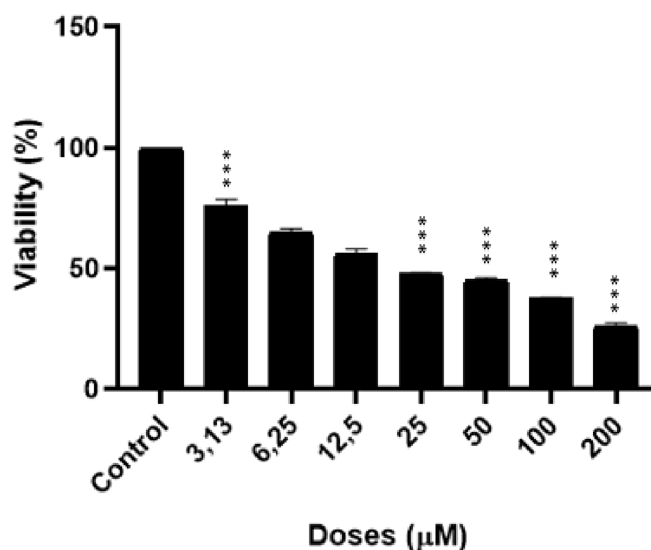

Figure 1: Viability percentages of A549 cells exposed to different ellagic acid concentrations ranging from 3.13-200 µM for 24 hours. ***$p<0.0001$

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B. Confocal Microscopic Results

Confocal images of acridine orange and phalloidin stained A549 cells treated with IC_{50} value of ellagic acid for 24 hours showed highly changed morphology when compared with the untreated A549 cells as shown in figure 2A and B. Untreated cells were with fusiform morphology and compact cytoskeleton and nuclei (Figure 2A) whereas ellagic acid treated cells showed chromatin condensation, holes on cytoskeleton and circular cell shape due to the shrinkage caused by ellagic acid (Figure 2B).

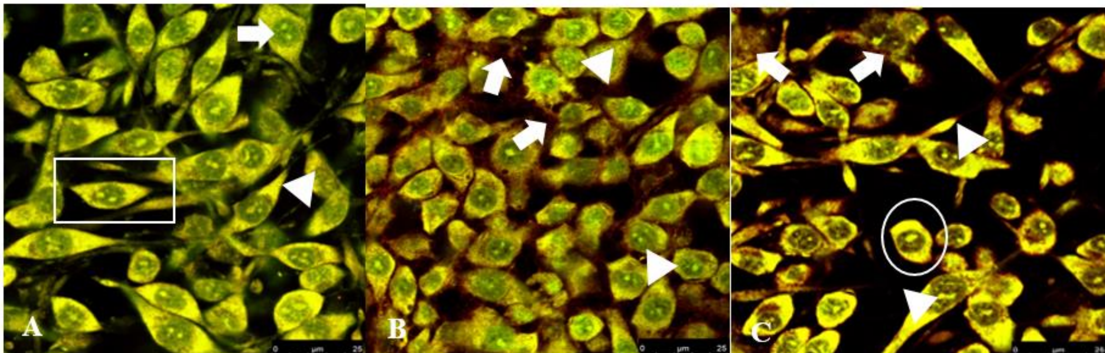

Figure 2: Confocal images of the untreated A549 cells (A): Arrow-normal nuclei, Arrowhead-normal cytoskeleton, Rectangle-fusiform cell shape and ellagic acid treated A549 cells (B, C): Arrowhead-chromatin condensation, Arrow-holes on cytoskeleton, Circle-shrunken cells.

C. TEM Results

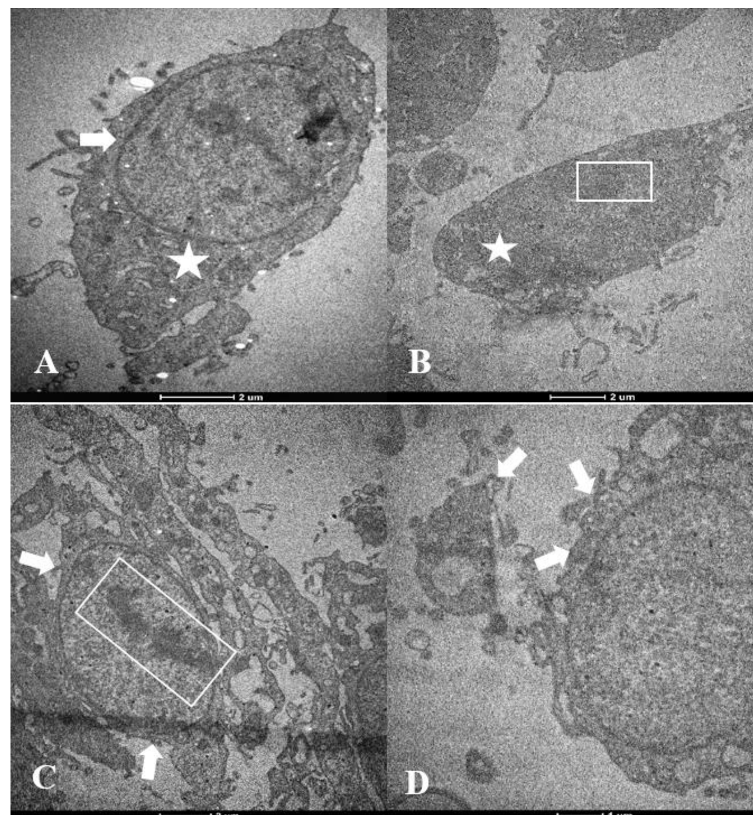

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Figure 3: TEM images of untreated and ellagic acid treated A549 cells. A: Arrow-cell membrane, asterisk-cytoskeleton. B: Rectangle-condensed chromatin, asterisk-granulated cytoplasm, C: Arrows-membrane blebbing, Rectangle-condensed chromatin. D: Arrows-membrane blebbing.

IV. DISCUSSION

In this study, the cytotoxic and growth inhibitory effects of ellagic acid on human non-small cell lung cancer cells, A549 were investigated. The data showed that ellagic acid application for 24 hours significantly inhibited cell viability in a dose-dependent manner. Our MTT findings underlined the cytotoxic effect of ellagic acid on A549 cells with an IC_{50} concentration 30 µM for its short-term application of 24 hours. The cytotoxicity on ellagic acid was found to be through causing changes on the cell morphology and ultrastructure indicating apoptosis. Morphological changes detected by confocal microscopic evaluation were found to be chromatin condensation, fragmentation on the cytoskeleton and nuclei. The changes detected on the A549 cells as damaged cell skeleton, cell shrinkage, nuclear fragmentation and other changes in the nucleus (Figure 2B and C) were considered to indicate apoptosis but further evaluations need to be performed. In addition, ultrastructural analysis performed using a TEM supported our confocal findings (Figure 3). In TEM assessment it was found that A549 cells exposed to 30 µM ellagic acid for 24 hours were with condensed nucleus, granulated cytoplasm, condensed chromatin, membrane blebbings (Figure 3B, C and D). Also, these ultrastructural changes were found to imply to ellagic acid-derived apoptotic cell death in A549 cells. In a similar study performed on C6 rat glioma cells, it was determined that the viability of the cells decreased at the 24 hours and 48 hours and was statistically significant as a result of application of 1µM concentration of ellagic acid ($p < 0.01$). They have reported that as a result of the application of 10 µM ellagic acid, no significant changes were observed in cell viability compared to the control group. They found that application of 100 µM ellagic acid was found to cause a statistically significant decrease in the proportion of live cells compared to the control group at 24 hours of application ($p<0.01$) [7]. Furthermore, in our previous study with ellagic acid on C6 rat glioma cells the IC_{50} value of the agent in short-term application of 24 hours was found to be 150 µM that is to high when compared to 30 µM IC_{50} value of this study [7]. This difference might be derived from the cell type used in the study.

Ellagic acid have been investigated for its antitumor properties on various in vitro studies. Study results imply to the anticancer effectiveness of ellagic acid for its usage as a drug for cancer treatment in clinics [8]. Moreover, the studies showed that ellagic acid reduces PI3K and AKT phosphorylation and also promotes A549 cell apoptosis that is the preferred way of death in cancer treatment. The ability of induction of programmed cell death of ellagic acid can be attributed to the effect of regulating apoptosis-related proteins [5]. Similarly, in this study results it was showed that ultrastructural and morphological changes of A549 cells caused by ellagic acid imply to apoptosis but clear determination of the cell death mode requires investigations with other apoptosis detection methods.

In conclusion, ellagic acid exerted valuable cytotoxicity on A549 cancer cell line in low doses that might be valuable data for further investigations in different cancer cell types and *in vivo* in order to discover an alternative agent for cancer treatment. According to our results, commercial ellagic acid caused structural changes in A549 cells by triggering apoptosis and led to high cytotoxic activity on these cells. Consequently, ellagic acid may be suggested for deeper investigation in a wide range of cancer cells comparatively with the normal cells and *in vivo* cancer models with an aim to produce an alternative agent or drug for the treatment of different cancers.

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