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ANTI-CANCER EFFECTS OF GRAPE SEED ON ISHIKAWA CELL CULTURE

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ABSTRACT

Cancer is a serious health problem. It still affects millions of people. In vitro cell culture is a method used to study the behavior of cells. The interest in grape seed is due to its high content of antioxidants in the form of proanthocyanidins. Its antioxidant capacity is greater than known antioxidants.

Ishikawa cells were cultured in complete DMEM/F-12 medium containing 100 units/mL-100 µg/mL of penicillin-streptomycin and 10% fetal bovine serum in 37 °C and 5% CO₂ incubator. After incubation of Ishikawa cells MTT (5 mg/mL) dye was added (20 µL/well) and plates were incubated again for two hours. Cell death mode triggered by grape seed extract on Ishikawa cells was analyzed by using the Annexin-V technique. To test the activation of caspases on Ishikawa cells exposed to grape seed extract Caspase 3/7 technique was used. For analysis of results one way variance analysis for multiple comparisons of GraphPad Prism 6.0 for Windows was used.

Grape seed extract showed its toxicity on Ishikawa cells in dose dependent manner in 24 and 48 hours of exposure. Annexin-V technique showed that grape seed extract induced apoptosis on Ishikawa cells. When compared to the control group. Ishikawa cells treated with grape seed extract imply to total apoptosis percentage of 22.90%. Based on this finding it can be concluded that grape seed extract has apoptosis inducing activity on Ishikawa cells in short application time.

Thus, grape seed extract worth to be elucidated for its further anticancer effects with deeper mechanistic studies in order to provide an option for designing novel anticancer drug as supplement or alternative to current chemotherapeutics.

Key Words-Grape seed extract, Ishikawa cell culture, Anti-cancer effects, MTT assay, Annexin-V, Caspase

1. INTRODUCTION

Cancer is a serious health problem. It still affects millions of people around the world. It is a disease in which certain cells in the body grow uncontrollably and spread to other parts of the body. It can start in almost any part of the human body. Endometrial cancer is the most common type of cancer in developed countries and the second most common type of cancer in developing countries. Five-year survival of patients with late-diagnosed endometrial cancer is below 30%. Therefore, it is very important to develop an appropriate

chemotherapeutic regimen for late-stage endometrial cancer¹.

In vitro cell culture is a method used to study the behavior of cells. A controlled environment is created in cell culture and this environment is protected from possible variations. Today, a wide variety of cell cultures have been developed. Cell cultures are used in areas such as basic cell biology, the effects of drugs and other chemicals, and vaccine production. The *in vitro* study of cellular activity has several advantages and disadvantages. The most important advantage of cell cultures is the consistency and reproducibility of the results obtained. In addition, cell cultures are working environments where physiological conditions can be precisely controlled. In these environments, the results are pretty clear. Although it is accepted that cell cultures do not fully represent processes in living tissue, it is currently considered the most precise and controlled method for research projects. The use of cell cultures in research is the first choice in areas such as the study of pathophysiology and therapeutic targets of various diseases and disorders^{2,3}.

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. For cell populations, total mitochondrial activity is related to the number of viable cells. Therefore, MTT is widely used to measure the *in vitro* cytotoxic effects of drugs on cell lines or diseased cells^{4,5}.

Mitochondria play a central role in apoptosis. Apoptosis is programmed cell death associated with caspase enzyme activity to maintain homeostasis in multicellular organisms. It is one of the most important intracellular events in the living cell. Caspase-3 and caspase-7 are cysteine-aspartic acid proteases that can directly perform apoptosis after caspase activation. Imaging of apoptosis in living cells provides great advantages, especially in drug research. One of the biochemical features of cells undergoing apoptosis is loss of plasma membrane asymmetry. As a result of this loss, a high amount of phosphatidylserine (PS) is released on the outer cell surface. Annexin V is a phospholipid-binding protein with a high affinity for PS and binds tightly to PS. Therefore, fluorescently labeled Annexin V can be used to detect PS released in apoptotic cells⁶⁻⁸.

A wide variety of studies over the years have identified the presence of potential agents in routinely consumed plant-based diets. These are mostly phytochemicals such as non-nutritive phenolics, glucosinolates, and terpenoids, which are divided into different classes according to their chemical structure. They are present in fruits, vegetables, nuts, and commonly consumed beverages such as tea and coffee. These phytochemicals show selective toxicity against cancerous or pre-cancerous cells. They have aroused scientific interest because they meet the basic requirements of an ideal chemopreventive agent, such as efficacy, oral route of administration, and acceptance by the target human population⁹.

Grape seed is an over-the-counter product used to contribute to a healthy life, widely consumed as a food supplement. The interest in grape seed is due to its high content of antioxidants in the form of proanthocyanidins. Its antioxidant capacity is greater than known antioxidants such as vitamin C and E¹⁰.

2. MATERIALS AND METHODS

Human endometrial adenocarcinoma Ishikawa cells were obtained from the American Type Culture Collection (Manassas, USA). Grape seed extract was obtained from Akcan Kimya (Turkey), fetal bovine serum (FBS), penicillin/streptomycin, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide (MTT), and Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) were purchased from Sigma-Aldrich (St. Louis, USA). Caspase 3/7 and Annexin-V Kits were from (Merck, Millipore, USA).

2.1 Culture of ishikawa Cells

Ishikawa cells were cultured in complete DMEM/F-12 medium containing 100 units/mL-100 µg/mL of penicillin-streptomycin and 10% fetal bovine serum in 37 °C and 5% CO₂ incubator. The test cells with confluency of 85% were passaged routinely per 3 days and used for experimentations.

2.2 MTT assay

Grape seed extract was diluted in DMSO to obtain a stock solution for further dilutions during the experimentation. The stock solution of grape seed extract was diluted with freshly prepared complete DMEM/F-12 medium and placed in 96 well test plates. Ishikawa cells were seeded (5×10^3 /well) in 96 well culture plates and incubated for 24 and 48 hours in 37 °C and 5% CO₂ incubator conditions. After incubation MTT (5 mg/mL) dye was added (20 µL/well) and plates were incubated again for 2 hours. After the second incubation, all of the solutions were aspirated from the plates and DMSO (200 µL/well) was added to dissolve the formazan crystals. Absorbances of the plates were read at 570 nm (n=3) on an ELISA reader (HTX Synergy, BioTek, USA). Obtained values were used for calculating the viability percentages and IC₅₀ values.

2.3 Annexin-V assessment

Cell death mode triggered by grape seed extract on Ishikawa cells was analyzed by using the Annexin-V technique. In this manner, Ishikawa cells were plated in six-well culture plates (5×10^5 cells/well) and exposed to IC_{50} dose of grape seed extract for 24 hours at 37 °C and 5% CO_2 incubator conditions. All cell samples were trypsinized and harvested in separate test tubes. Collected cells were washed in buffer (PBS). Annexin-V dye (100 μ L/sample) was added and further incubated for 20 minutes at dark at room temperature (Muse® Annexin-V and Dead Cell Assay Kit). Ishikawa cells were analyzed with Muse™ Cell Analyzer (Merck, Millipore, Hayward, California, USA).

2.4 Caspase 3/7 activation assessment

To test the activation of caspases on Ishikawa cells exposed to grape seed extract Caspase 3/7 technique was used. Ishikawa cells exposed to IC_{50} value of grape seed extract for 24 hours in six-well plates and untreated Ishikawa cells cultured in the same conditions and density (5×10^5 /well) with test cells were harvested by trypsinization and washed in PBS. Caspase 3/7 working solution and 7-ADD solutions were added to all sample groups according to the user manual of the manufacturer of the caspase 3/7 kit (Merck, Millipore, Hayward, California, USA). Samples were analyzed on a cell analyzer (Muse™ Cell Analyzer, Merck, Millipore, Hayward, California, USA).

2.5 Statistical analysis

For analysis of results one way variance analysis for multiple comparisons of GraphPad Prism 6.0 for Windows was used.

3. RESULTS

3.1 MTT findings

Grape seed extract showed its toxicity on Ishikawa cells in dose dependent manner in 24 and 48 hours of exposure (Figure 1). The highest decrease was detected at 1 mg/mL of grapes seed extract both for 24 and 48 hours of application. IC_{50} values of grape seed extract were detected to be 0.09 mg/mL for both exposure times. Moreover, the obtained data imply to the efficacy of grape seed extract in growth inhibition and cytotoxicity both for application periods of 24 and 48 hours.

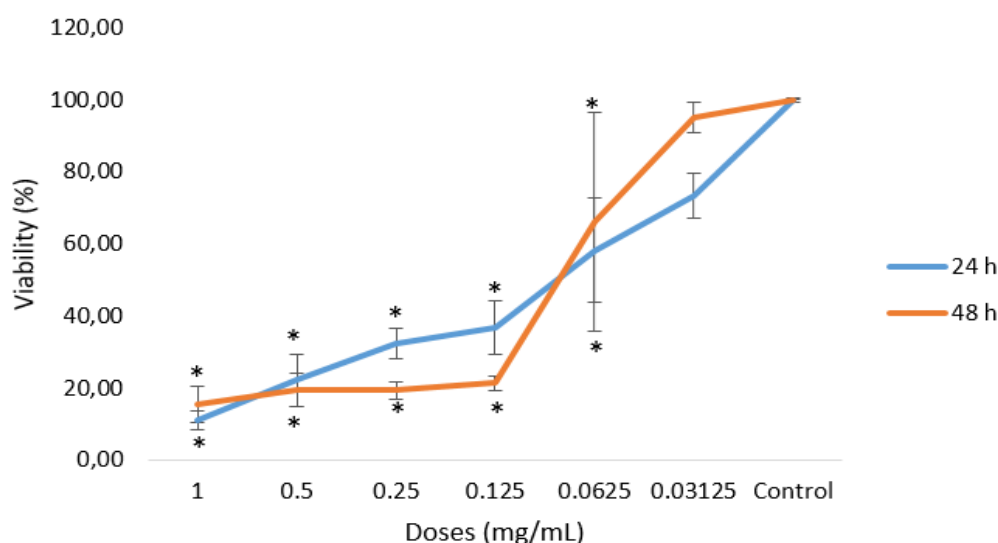


Fig. 1: Growth inhibition activity of grape seed extract on Ishikawa cells. IC_{50} dose of grape seed extract: 0.09 mg/mL for 24 and 48 hours. (*: $p < 0.5$)

3.2 Annexin-V results

Apoptotic profiles of Ishikawa cells analyzed by annexin-V technique showed that grape seed extract induced apoptosis on Ishikawa cells. When compared to the control group (Figure 2A) Ishikawa cells treated with grape seed extract imply to total apoptosis percentage of 22.90% (Figure 2B). Based on this finding it can be concluded that grape seed extract has apoptosis inducing activity on Ishikawa cells in short application time.

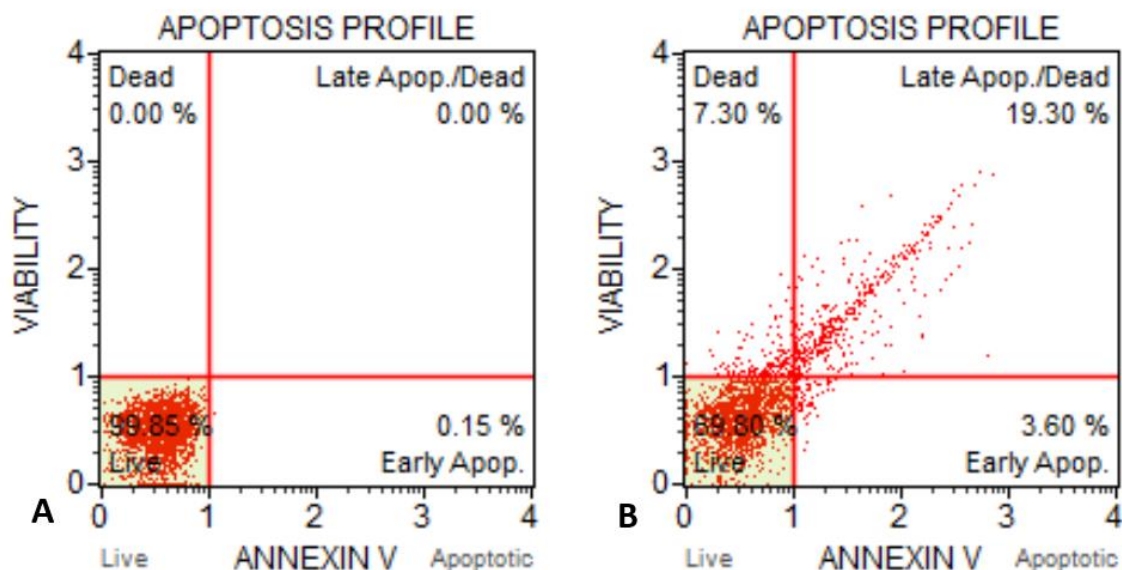


Fig. 2: Apoptosis profiles of Ishikawa cells

Note: 2A. Control Ishikawa cells; Live cells 99.85% and 0.15% early apoptotic cells were detected. Percentages of late apoptotic and dead cells were detected as 0.00%. 2B. Ishikawa cells exposed to IC₅₀ value of grapes seed extract for 24 hours. 69.80% of cells were live. The percentages of dead, early apoptotic and late apoptotic cells were detected to be 7.30%, 3.60% and 19.30% respectively

3.3 Caspase 3/7 results

Cells undergo apoptosis *via* various pathways and by the influence of intracellular or extracellular factors. Caspases are major elements of those apoptotic pathways that initiate apoptosis irreversibly by their activation. Herein, the total percentage of activated caspases was found to be 6.50%. In control cells this percentage was detected as 3.05% (Figure 3). Flow cytometry findings underlined that grape seed extract induced apoptotic cell death in Ishikawa cells and based on the caspase 3/7 analysis results it was clearly detected that the triggered apoptosis is caspase-dependent.

Based on the data obtained within the scope of this study it is concluded that grape seed extract exerts cytotoxicity on human endometrial adenocarcinoma cells in low doses being anti-proliferative and proapoptotic in dose-dependent manner. Thus, grape seed extract worth to be elucidated for its further anticancer effects with deeper mechanistic studies in order to provide an option for designing novel anticancer drug as supplement or alternative to current chemotherapeutics.

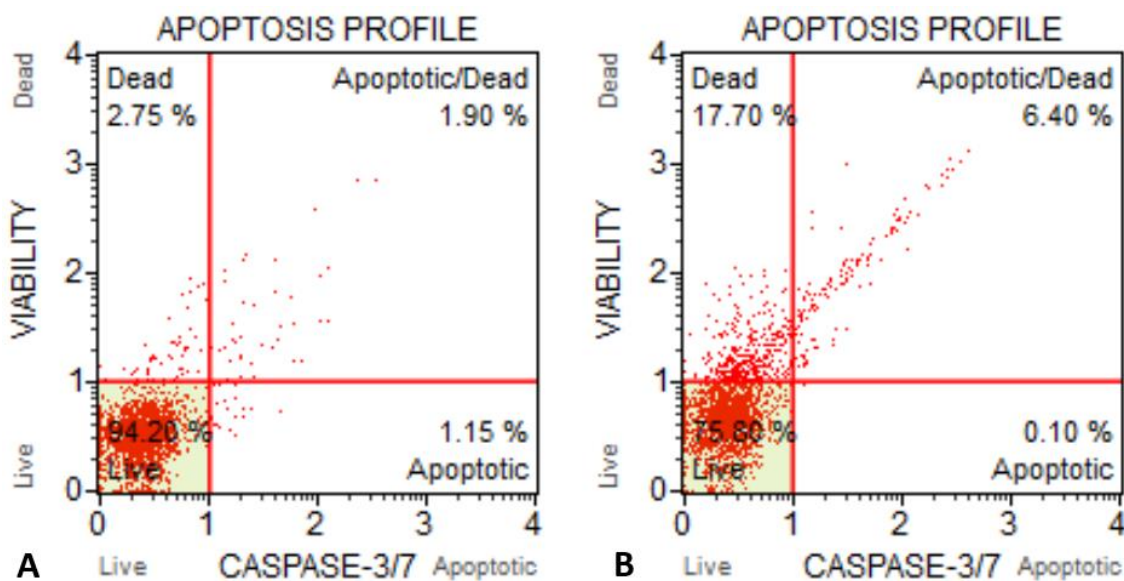


Fig. 3: Caspase-dependent apoptosis profiles of Ishikawa cells

Note: 3A. Untreated Ishikawa cells (94.20% Live, 2.75% Dead, 1.90 Apoptotic/Dead and 1.15% Apoptotic cells). 3B. Ishikawa cells treated with IC₅₀ concentration of grape seed extract for 24 hours (75.80% Live, 17.7% Dead, 6.4% Apoptotic/Dead and 0.10% apoptotic cells)

4. DISCUSSION

Grape seed extract is a mixture of various compounds of polyphenols and phenolic acids. Their consumption is safe. It is considered to have health benefits. The extract-derived anti-tumor activity acts through a variety of biological mechanisms and cellular targets. It causes inhibition of cell growth and apoptosis in various cancer cell lines. These effects are modulated at the molecular level. Anti-cancer activity from grape seed extract is mostly based on the increase of reactive oxygen species. This increase is followed by the upregulation of several key molecular pathways, including kinases, nuclear factor kappa B, cytoskeletal proteins, and metalloproteinases. Promising results have been obtained on grape seed extract *in vitro* as well as in animal studies. It is thought to be a source of potential new pharmacological molecules and an important opportunity for clinical research¹¹⁻¹³.

Flavonoids contain various classes of compounds such as flavones, flavonols, and anthocyanins. Each class is different from the other. Flavonoids are generally found in nature as glycosides. Grape seed has both phenolic acid and flavonoid content. This content constitutes 60%-70% of the dry extract. Grape seeds also contain resveratrol and anthocyanidin, but their density is less^{14,15}.

It has been shown that grape seed extract components have antioxidant, anti-inflammatory, antiaggregant and antimicrobial effects. These properties are attributed specifically to more potent ingredients such as gallic acid, procyanidins and quercetin. Gallic acid and procyanidins generally make up about 80% of the dry extract. The effects of grape seed extract are often attributed to these molecules. However, since some functions are provided by synergistic interactions between different components, the contribution of other molecules cannot be ignored. For example, the antioxidant effects of the extract can be explained by the sum of the antioxidant activities of each component. Similarly, even though it is thought that the anticancer effects are generally performed by procyanidins and epigallo-catechin-3-gallate, the anticancer effect is higher than the sum of each component¹³⁻¹⁶.

In this case, the effect obtained from grape seed extract is greater than the effects of the components contained in the extract alone. This indicates that there is a synergism between the components. In this respect, it is more meaningful to examine the grape seed extract as a whole when examining its health effects, and the results will be more reliable. Considering this issue, grape seed extract was used as a whole in our research.

When the data of some studies are examined, it is seen that important results have been obtained. The PI3K/Akt pathway plays a crucial role in mammalian cell survival signals and has been shown to be activated in various cancers. Phosphorylated PI3K and Akt are thought to be key elements in modulating kinase activation and NF- κ B-dependent pathways. Grape seed extract has been shown to

reduce PI3K levels and Akt phosphorylation, increasing Akt degradation. PI3K/Akt phosphorylation is negatively regulated by 'chromosome 10 deleted phosphatase and tensin homolog' (PTEN), a lipid phosphatase that inhibits it. The absence of PTEN is strongly associated with PI3K/Akt activation in tumor cell lines. Grape seed extract significantly reduces PTEN phosphorylation and increases its negative regulation on the PI3-K pathway. In addition, grape seed extract suppresses Akt-related effects on CREB, NFkB, BAD and Bcl-2, promoting a general pro-apoptotic effect on cancer cells¹⁷⁻²⁰.

Mitogen activated protein kinase (MAPK) signaling pathway is an important regulator of transcriptional factor activities. It affects various extracellular stimuli by controlling the activities of MAPK transcription factors and plays a role in cancer development and progression. It has been reported by some studies that grape seed extract increases the activation of JNK and p38MAPK through increased intracellular calcium. In contrast, p38MAPK increases apoptosis through Bcl-2 inactivation, caspase increase and mitochondria depolarization. This effect of p38MAPK has been associated with an increase in intracellular calcium and is thought to participate in the enhancement of grape seed extract-induced apoptotic effect on cancer cells²¹⁻²⁴.

Some studies have shown that inflammation modulators are functionally associated with tumor development. Prostaglandins, one of the most important of these modulators, are produced in abundance by the metabolic conversion of arachidonic acid by COX-2. Prostaglandins produced are known to play a role in the development of many malignancies. NF-kB increases COX-2 expression. Grape seed extract has been proven to down-regulate the expression of NF-kB and COX-2. Activator protein-1 (AP-1) levels, which increase COX-2 expression, are also down-regulated by grape seed extract. Data from various studies show that grape seed extract inhibits cancer development by reducing the level of prostaglandins²⁵⁻²⁸.

Data have been reported proving that grape seed extract induces apoptosis in cancer cells by down-regulating anti-apoptotic proteins. These effects are due to inhibition of the PI3K pathway and modulation of p38MAPK and ERK, as mentioned above. This pro-apoptotic effect is highly specific, as normal cells are generally insensitive to grape seed extract. It has been reported that grape seed extract induces apoptosis in p53-expressing cancer cells more than others. Some components in the extract recognize p53 as a specific target and bind to integrin $\alpha\beta3$, leading to p53 activation. However, there are also studies showing that the cytotoxic effect of grape seed extract is independent of the p53 status of cancer cell lines. One of the most common genetic defects found in cancers is the mutation of the TP53 gene, which encodes the p53 protein. Therefore, the observation that the cytotoxic effect is independent of p53 status is very important²⁹⁻³².

5. CONCLUSION

In conclusion, the data obtained in our research is supported by many studies. It is thought that it is important to reveal the effect shown on proteins such as caspases and annexin in our study and to support this with MTT analysis. The fact that the grape seed extract was examined as a whole in our study is also important in terms of revealing the synergistic effects of the components it contains. It is considered that the comparative examination of seed extracts obtained from different grape species will contribute to the subject in the following studies.

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