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Evaluation of antitumoral effect of mistletoe fruit extract on Ehrlich ascites tumor cells with muse cell analyzer and argyrophilic nucleolar organizer region staining method

Original Study

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Abstract

Introduction. Mistletoe has been used alone or as a complementary therapy in the treatment of different diseases for years. In this study, the antitumoral effect of mistletoe fruit extract on Ehrlich ascites tumor (EAT) cells was evaluated.
Materials and Methods. EAT cells from preformed stock mice were transferred to culture dishes containing 5-fluorouracil (5-FU) and mistletoe extracts at different doses (100, 200, 400, and 800 µg/ml). These cells were incubated at 37 °C in an environment with 95% humidity and 5% CO2. At the end of the incubations, the apoptosis status of the cells, cell cycle, mitochondrial membrane potential, and proliferation status with the argyrophilic (Ag) nucleolar organizer region staining (NORs) method were evaluated.
Results. As a result, it was observed that the mistletoe fruit extract and 5-FU induce apoptosis of EAT cells. It was concluded that the 5-FU substance arrests the cell cycle at the G0/G1 stage, while the mistletoe arrests the cell cycle at the S and G2/M stages. The depolarization rate of the mistletoe treated cells was higher. As a result of the evaluation made with the AgNORs method, it was seen that mistletoe and 5-FU could be effective in reducing the proliferation of EAT cells.
Conclusions. It was seen that mistletoe fruit extract could be effective in stimulating the apoptosis and depolarization of cancer cells. The results of other studies in the literature and our study support each other. It was concluded that the mistletoe plant may be useful in cancer treatment.

Keywords

mistletoe • *Viscum album l.* • Ehrlich ascites tumor • apoptosis • cell proliferation • AgNORs

1. Introduction

Cancer, which has a significant share among the causes of death due to disease, is still being studied by many researchers with curiosity about the disease and its treatment [1]. Different treatment methods are used in cancer, including chemotherapy and radiotherapy. The treatment method used varies depending on the type of cancer and the condition of the patient [2]. In complementary therapy, the main goals are to reduce the symptoms caused by the disease and the side effects caused by the drugs and to provide physical and psychological support [3, 4]. Overall survival of cancer patients using medicinal herbs as complementary therapy is prolonged [5, 6]. In line with this goal, medicinal and aromatic plants containing sufficient amounts of phenolic substances are also used as antioxidant sources in the treatment of cancer and other diseases [7, 8].

Mistletoe, one of the medicinal and aromatic plants, is included in the genus viscum, *Loranthaceae* family (*viscum album L.*). There are around 100 species (species/sp.) in all geographies [9]. *Viscum album L.* contains compounds such as polyholosides, lectins, viscotoxins, flavonoids, alkaloids, nitrogenous compounds, phenylpropane, and lignans. Among these compounds, especially polyholosides, lectins, and viscotoxins are reported to have anticarcinogenic effects [10, 11].

Evaluating the cellular events that occur in cancer allows a better interpretation of the course and treatment of the disease. Changes in cellular events, such as apoptosis of cancer cells, cell cycle, and mitochondrial membrane potential, are automatically

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evaluated with the Muse Cell Analyzer device and kits suitable for this device. Muse™ Annexin V & Dead Cell Assay kit is used to detect phosphatidylserine (PS) in the outer membrane of apoptotic cells. The Muse ™ Cell Cycle Assay kit is based on propidium iodide (PI) -based staining of DNA content to determine the amount of cells in different stages of the cell cycle (G0/G1, S, G2/M). The Muse ™ Mitopotential Assay kit is used to detect changes in mitochondrial membrane potential and 7-amino actinomycin (7-ADD), an indicator of cell death, using the Mitopotential dye [12, 13]. In addition, the proliferation of cancer cells can be evaluated with argyrophilic nucleolar organizing region staining (AgNORs). On chromosomes, there are regions called "Nucleolar Organizing Region (NOR)" consisting of ribosomal DNA (rDNA) and proteins. These regions (NOR) can be dyed with silver (Ag) when they are active and therefore they are called AgNOR. AgNORs are used to evaluate the proliferation status of cancer cells [14].

In this study, the antitumoral effect of mistletoe fruit extract against Ehrlich ascites tumor (EAT) cells was evaluated in vitro. This effect of the mistletoe fruit extract was compared with that of the 5-FU chemotherapeutic agent. In the evaluation, variables such as the viability of EAT cells, apoptosis status, the stage at which the cell cycle arrested, mitochondrial membrane potentials, and the amount of nucleolar organizer region (NOR) stained with silver (Ag) were analyzed.

2. Materials and Methods

In the present study, the applications performed on experimental animals were evaluated in terms of animal rights and welfare, and their suitability in terms of animal experiments ethics was approved by the Erciyes University, Experimental Animals Local Ethics Committee, decision number 19/053, dated 06.03.2019. The maintenance-feeding and application processes and in vitro cell culture studies of the stock animals created in the experimental stage were carried out at Betül – Ziya EREN Genome and Stem Cell Center. The experimental process in this study is given in Figure 1.

2.1. Mistletoe fruit extract

Mistletoe growing on apricot trees in the vicinity of Ali Mountain in the Talas district in Kayseri province (Turkey) were collected and the species determination was made at Erciyes University, Faculty of Science, Department of Biology. This plant specimen was recorded as a herbarium entry with the HS1615 sample code. The fruits of the collected mistletoe plant were laid on blotter papers under room conditions and left to dry. 250 ml of distilled water was added to 10 g of dried mistletoe fruit and kept for 24 hours; extracts were obtained after four times shaking water bath at room temperature. The extracts obtained were combined and concentrated in a rotavapor (37-38°C) under vacuum. Dry extracts obtained after lyophysis were diluted with DMEM (Dulbecco's Modified Eagle medium). Then, it was passed through 0.45 µm and 0.22 µm sterile injector filters, respectively.

2.2. Total phenolic and flavonoid contents

Total phenols were estimated as gallic acid equivalents, expressed as mg of gallic acid/$g_{extract}$. 100.0 µL of the sample was transferred in a 10.0 mL volumetric flask, to which 500.0 µL undiluted Folin–Ciocalteu reagent was added subsequently. After 1 min, 1.5 mL 20% (w/v) Na_2CO_3 was added and the volume was made up to 10.0 mL with H_2O. After 2 h incubation at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. The data are presented as the average of triplicate analyses. Total flavonoid analysis of the extracts was made by modifying the method used by Zhishen et al [15, 16]. Accordingly, 1 mL extract with 0.3 mL of 5% $NaNO_2$ solution mixed at t = 0 min, after addition of 0.3 mL of a 10% $AlCl_3.6H_2O$ solution at t = 5 min., 2 mL of 1 M NaOH solution and 2.4 mL of water was added at t = 6 min. At 510 nm, the absorbance against the blind was measured. The total flavonoids contained in the extracts were calculated as catechin equivalents in mg CA/$g_{extract}$. Calibration curve of catechin was prepared in the same manner using ethanol.

2.3. Creating a stock mouse with EAT cells

Male mice, 8-10 weeks old, Balb/C race, weighing 25-30 g were used in the study. The mice were housed at a constant temperature of 21°C for the duration of the study, in rooms with automatic monitoring of humidity and a 12 hour light/dark cycle. The animals were provided normal pellet-type feed and tap water. EAT cells frozen in cryo tubes containing 10% dimethyl sulfoxide (DMSO) and stored at -80°C were kept at normal room temperature to dissolve. After checking the viability of the dissolved cells, ascites fluid containing EAT cells was injected intraperitoneally with the help of a sterile injector. After the application, the animal was monitored daily (Figure 1).

2.4. Cell culture

The liquid containing EAT cells was drawn from the stock mouse intraperitoneally, and 24-well plates were used in the culture process. Dulbecco's Modified Eagle Medium (DMEM) containing 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture was used as the medium (Gibco™ DMEM is manufactured at a cGMP compliant facility, located in Paisley, Scotland, UK/Catalog no: 41966029). In the experiment, the antitumoral effect of mistletoe fruit extract on EAT cells was tested at different doses in the range of 100-800 µg/ml. In addition, this effect was compared with the control and 5-FU (15 µg/ml) groups. Cells were taken to the incubator at 37 °C with 95% humidity and 5% CO2 for 24, 48, and 72 hours (Fig. 1).

2.5. Annexin V and dead cell assay

EAT cells were cultivated in 24-well plates with $1x10^5$ cells per well and left to incubate for 24, 48, and 72 hours. At the end of the incubation periods, each well in the well plate was taken into an Eppendorf tube with the name of its group and centrifuged at 300 g for 5 minutes. After removal of the supernatant, it was washed with phosphate buffered saline (PBS). Then, 1% of 100 µl FBS and 100 µl the Muse® Annexin V & Dead Cell Kit (Catalog Number: MCH100105) were added. Then it was left to incubate for 20 minutes at room temperature in the dark. Samples were analyzed automatically with the Muse® Cell Analyzer (Merck-Millipore) device according to the manufacturer's instructions.

2.6. Cell cycle assay

EAT cells were cultivated in 24-well plates with $1x10^6$ cells per well and left to incubate for 24, 48, and 72 hours. At the end of the incubation periods, each well in the well plate was taken into an Eppendorf tube with the name of its group and centrifuged at 300 g for 5 minutes. Afterwards, washing was done with PBS kept at -20°C and 70% ethanol kept at -20°C was added. All tubes were kept at -20 °C overnight. After one night, the tubes were centrifuged at 300 g for 5 minutes, the supernatant was discarded and after washing with PBS, 200 µl of the Muse® Cell Cycle Kit (Catalog Number: MCH100106) was added. Tubes were left to incubate for 30 minutes at room temperature in the dark. Samples were analyzed automatically with the Muse® Cell Analyzer (Merck-Millipore) device according to the manufacturer's instructions.

2.7. Mitopotential assay

EAT cells were cultivated in 24-well plates with $1x10^5$ cells per well and left to incubate for 24, 48, and 72 hours. At the end of the incubation periods, each well in the well plate was taken into an Eppendorf tube with the name of its group and centrifuged at 1200 rpm for 5 minutes. Washing was done with PBS. Subsequently, the Muse® Mitopotential kit (Catalog Number: MCH100110) was used. Then 1 ml of mitopotential assay buffer liquid was added to the tubes, the tubes were vortexed. 100 µl of each tube was transferred to a new tube; 95 µl of standard mitopotential solution was added to the tubes and they were left in the incubator at 37°C for 20 minutes. Then, 5 µl of 7-ADD reagent was added to each tube and left for 5 minutes incubation in the dark at room temperature. Samples were analyzed

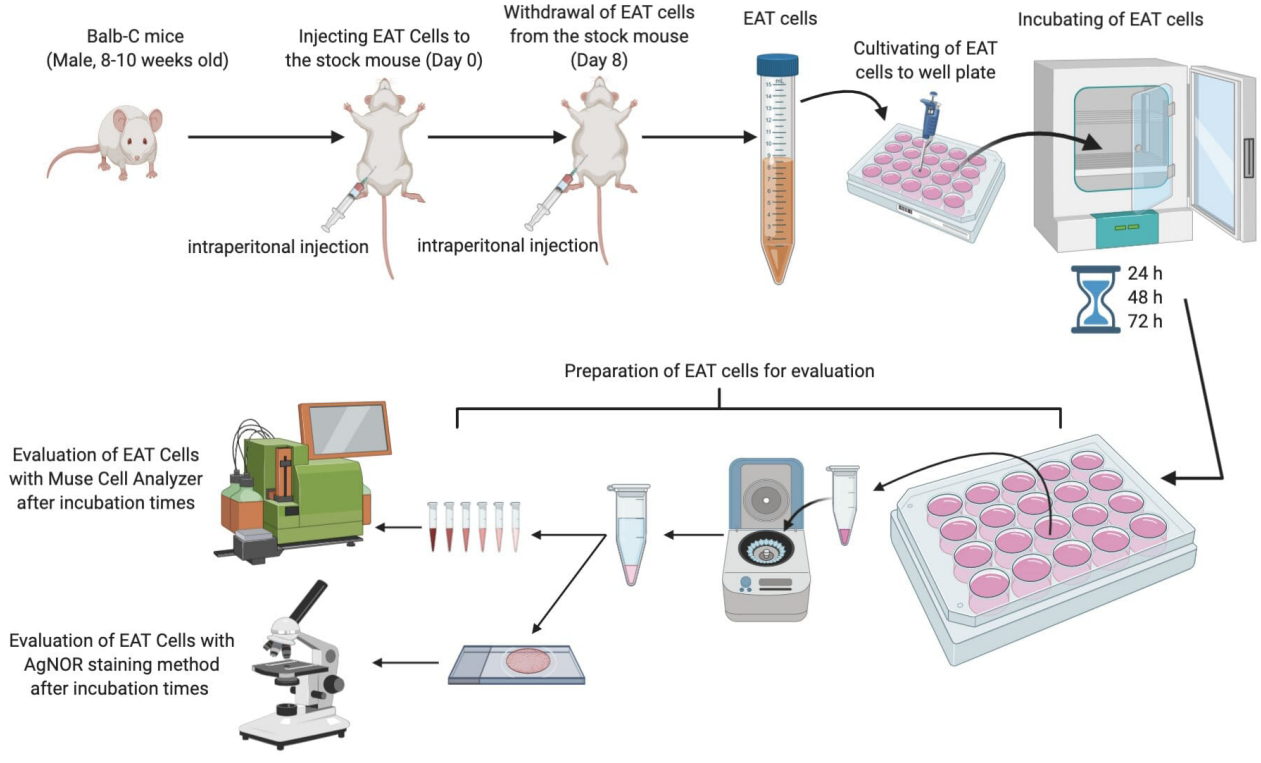

Fig. 1. Experimental processes in this study

automatically with the Muse® Cell Analyzer (Merck-Millipore) device according to the manufacturer's instructions.

2.8. AgNOR staining

At the end of the incubation periods, each well in the well plate was transferred to a tube with the name of its group. The fixative containing acetic acid (Merck, 247K18855556) – methanol (Merck, 502K5275408) in a ratio of 1: 3 was added to the cells and kept for 15 minutes. Then, after the cells were centrifuged, the supernatant was discarded and the remaining cells were vortexed and spread on slides kept at -20°C. Slides with fixed cells were allowed to dry at room temperature. Ag-NOR staining was performed on the dried slides by preparing a 50% Silver-Nitrate (Merck, 30474310228) solution containing 1% formic acid (Merck, K39189264836) and 2% gelatin (Merck, 39166778-839) according to Howell and Black (1980) [17]. AgNOR stained cells were examined under a light microscope (BX53, Olympus) and photographed with a digital camera (SC50, Olympus). Images of the cells were transferred to image processing software (ImageJ version 1.47t, National Institutes of Health, Bethesda, Maryland, USA). By evaluating cell nuclei, both the total AgNOR area (TAA/NA) and the average AgNOR number per nuclear area were calculated using the "free hand selections" tool.

2.9. Statistical analysis

Statistical Package for Social Sciences (SPSS, for Windows) version 23.0 program was used for all statistical analysis. With the Shapiro-Wilk test, it was observed that the data belonging to the groups were normally distributed. Since there is a normal distribution in the data, one-way ANOVA test was used in multiple comparisons between groups; the post-hoc Tukey test was used for paired group comparison. Statistically, a value of $p < 0.05$ was considered significant.

Table 1. Total phenolic and flavonoid contents of mistletoe fruit

	Total phenol ($mgGAE/g_{extract}$)	Total flavonoid ($mgCAE/g_{extract}$)
Mistletoe fruit	6.24±2.26	4.82±1.36

GAE: Gallic acid equivalents; CAE: Catechin equivalents.

3. Results

3.1. Total phenolic and flavonoid contents

Viscum album l. fruit total phenolic and flavonoid contents are shown in Table 1.

3.2. Muse® Cell Analyzer results

The raw graphics taken from the Muse Cell Analyzer device were interpreted and the results were evaluated (Fig. 2). The viability and apoptosis profiles, the stage in which the cell cycle arrests, and mitochondrial membrane potentials of EAT cells were evaluated.

3.3. Annexin V and dead cell assay

In this assay, the viability of the cells, early apoptosis, late apoptosis, and total apoptosis status were evaluated (Fig. 3).

In the data obtained from the Muse Cell Analyzer, when the live cell counts were evaluated, there was a significant decrease ($p < 0.05$) in the 5-FU group and all the mistletoe groups compared to the control group at the end of the 24-hour incubation. At the end of the 72-hour incubation, the viable cell count of the 5-FU group and the 100, 200, and 800 µg/ml mistletoe groups were significantly lower ($p < 0.05$) than the control group (Fig. 3A).

When the data at the end of the 24-hour incubation were evaluated, it was seen that the early apoptotic cell count was significantly higher in the 5-FU and 100, 200, 400 µg/ml mistletoe groups compared to the control group ($p < 0.05$). Early apoptotic

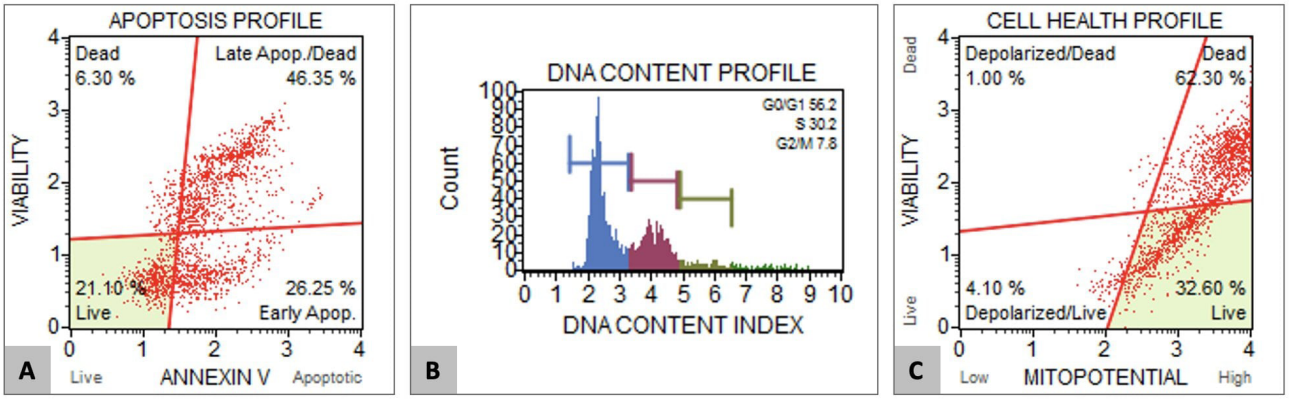

Fig. 2. The raw graphics taken from the Muse® Cell Analyzer. A: Muse® Annexin V and Dead Cell Assay, B: Muse® Cell Cycle Assay, C: Muse® Mitopotential Assay

cell count was significantly highest in the 5-FU group ($p < 0.05$). At the end of 48 hours, a significant decrease was observed in the early apoptotic cell count in the 400 µg/ml and 800 µg/ml mistletoe groups compared to the control group ($p < 0.05$), while the change in the other groups was insignificant ($p > 0.05$). After 72 hours, there was a significant increase ($p < 0.05$) in the early apoptotic cell count of only the 800 µg/ml mistletoe group compared to the control group (Fig. 3B).

When the 24-hour data were evaluated, it was seen that there was a significant difference between the groups ($p < 0.05$), but there was no significant difference in terms of late apoptotic cell count in the 5-FU and mistletoe groups compared to the control group ($p > 0.05$). At the end of 48 hours, there was a dose-dependent significant increase ($p < 0.05$) in late apoptotic cell count of 200, 400, and 800 µg/ml mistletoe groups compared to the control group. At the end of 72 hours, there was a significant increase ($p < 0.05$) in the late apoptotic cell count of the other groups compared to the control group (Fig. 3C).

When the 24-hour data were evaluated, a significant increase was observed in the total apoptotic cell count of the 5-FU, 100 µg/ml, and 400 µg/ml mistletoe groups, compared to the control group ($p < 0.05$). Considering the difference between the groups after 24 hours of incubation, it was seen that there was no significant difference between the 5-FU group and the mistletoe groups ($p > 0.05$). At the end of the 72-hour incubation, it was observed that the total apoptotic cell count of the 5-FU and 800 µg/ml mistletoe groups were significantly higher ($p < 0.05$) compared to the control group (Fig. 3D).

3.4. Cell cycle assay

In this assay performed with the Muse Cell Analyzer device, the data of the cell percentages at the G0/G1, S and G2/M stages were evaluated at the end of the incubation period (Fig. 4).

When the data at the end of 24 and 48 hours were evaluated, it was seen that the number of cells at the G0/G1 stage was significantly higher in the 5-FU group compared to the control group ($p < 0.05$). In the 72-hour data, this value was found to be significantly higher ($p < 0.05$) in the 5-FU group and the 200 µg/ml mistletoe group compared to the control group (Fig. 4A).

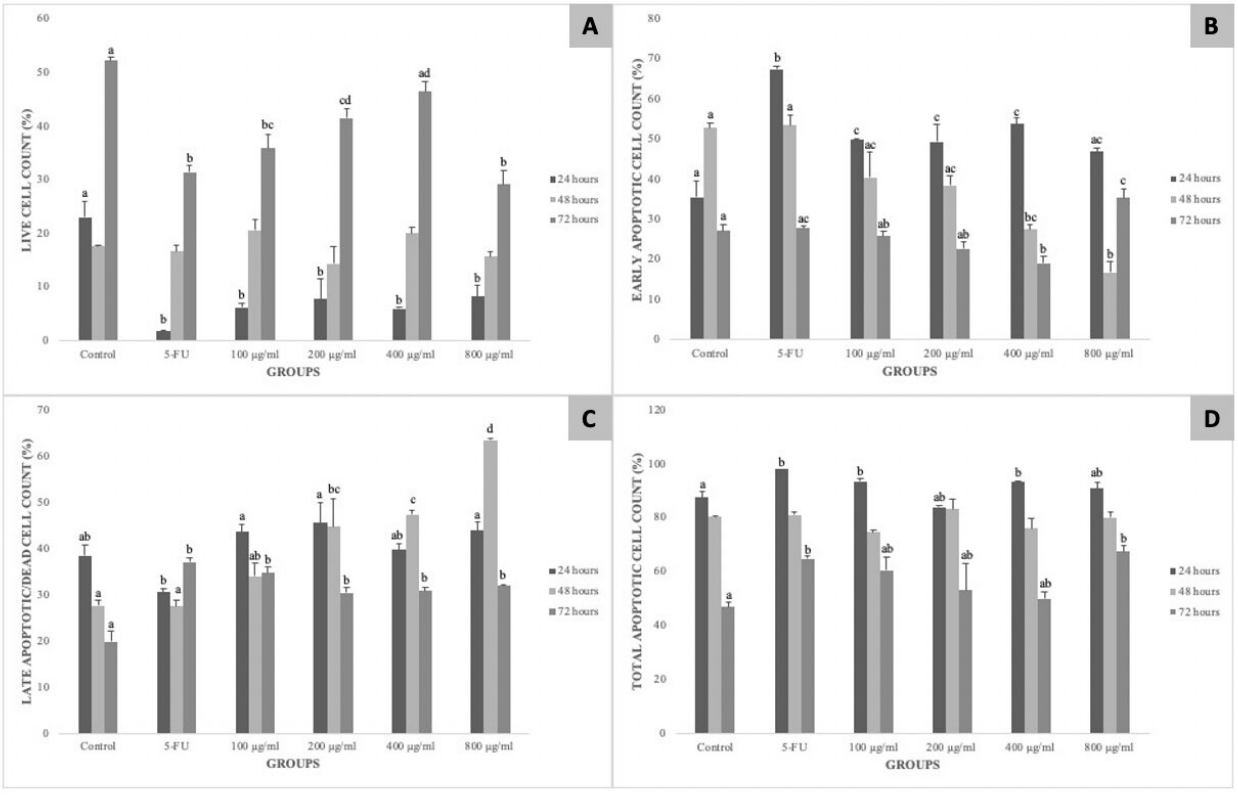

Fig. 3. Data of the annexin V & dead cell assay at the end of 24, 48, and 72 hours of incubation. $p<0.05$ was considered statistically significant. Data are expressed as mean ± SEM (standard error of mean). There is no statistically significant difference between the groups containing the same letter ($p > 0.05$). A: Live cell count (%), B: Early apoptotic cell count (%), C: Late apoptotic cell count (%), D: Total apoptotic cell count (%). 5-FU: 5-fluorouracil; 100, 200, 400, 800 µg/ml: Mistletoe groups

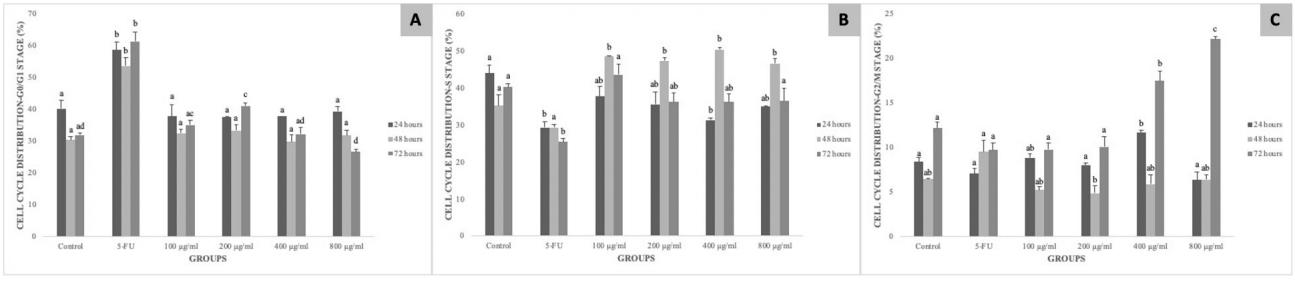

Fig. 4. Data of the cell cycle assay at the end of 24, 48, and 72 hours of incubation. p <0.05 was considered statistically significant. Data are expressed as mean ± SEM. There is no statistically significant difference between groups containing the same letter (p> 0.05). A: Cell cycle distribution-G0 / G1 stage (%), B: Cell cycle distribution-S stage (%), C: Cell cycle distribution- G2 / M stage (%). 5-FU: 5-fluorouracil; 100, 200, 400, 800 µg/ml: Mistletoe groups

At the end of 24 hours, the number of cells at the S stage was found to be significantly lower in the 5-FU and 400 µg/ml mistletoe groups compared to the control group (p <0.05). According to the 48-hour data, this value was significantly higher (p <0.05) in all mistletoe groups compared to the control group. At the end of 72 hours, this value was significantly (p <0.05) lower in the 5-FU group compared to the control group (Fig. 4B).

At the end of the 24-hour incubation, only the 400 µg/ml mistletoe group had a significantly higher number of cells at the G2/M stage compared to the control group (p <0.05). There was no significant difference between the control group and other groups in terms of the number of cells at the G2/M stage in the 48-hour data (p> 0.05). At the end of 72 hours, this value was found to be significantly higher (p <0.05) only in the mistletoe group of 800 µg/ml compared to the control group (Fig. 4C).

3.5. Mitopotential assay

Percentage values of depolarized live, depolarized dead, and total depolarized cells were compared with the applied test. The raw graphics taken from the Muse Cell Analyzer device were interpreted and the results were evaluated (Fig. 5).

As a result of the evaluation, there was a significant increase (p <0.05) in the live depolarized cell count only in the 800 µg/ml mistletoe group compared to the control group in the 24 and 72 hour data (Fig. 5A). In terms of the amount of depolarized dead cells, there was a significant increase (p <0.05) in the dead depolarized cell count in only 800 µg/ml mistletoe group compared to the control group at the end of 48 hours. At the end of 72 hours, the dead depolarized cell count in only the 100 µg/ml mistletoe group was significantly higher than the control group (p <0.05) (Figure 5B). In terms of the total depolarized cell count, there was a significant increase (p <0.05) in the mistletoe group only at 800 µg/ml compared to the control group at 24 and 72 hours of incubation (Fig. 5C).

3.6. AgNOR staining results

Image examples of AgNOR staining cells are shown in Figure 6. The mean AgNOR number (Table 2) and TAA/NA (Table 3) values are shown in the tables.

When the data of 24, 48, and 72 hours of incubation were evaluated in terms of mean AgNOR number and TAA/NA value, it was seen that there was a significant difference between the groups (p <0.05). When looking at the data from the 24-hour in-

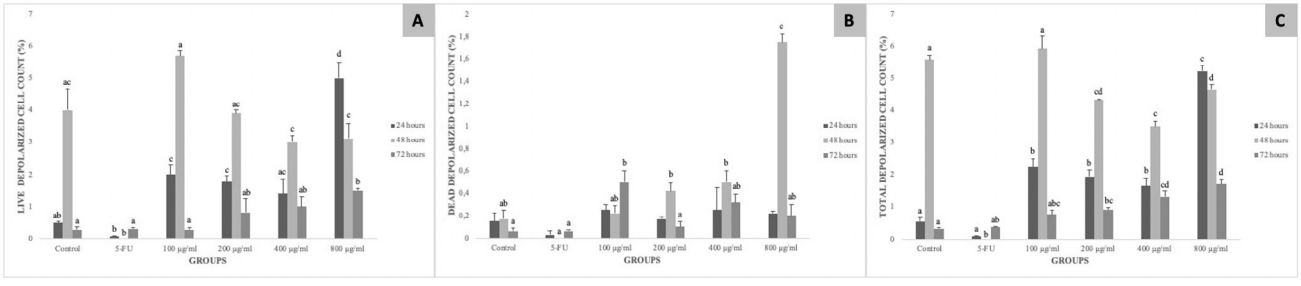

Fig. 5. Data of the mitopotential assay at the end of 24, 48, and 72 hours of incubation. p <0.05 was considered statistically significant. Data are expressed as mean ± SEM. There is no statistically significant difference between the groups containing the same letter (p> 0.05). A: Live depolarized cell count (%), B: Dead depolarized cell count (%), C: Total depolarized cell count (%). 5-FU: 5-fluorouracil; 100, 200, 400, 800 µg/ml: Mistletoe groups

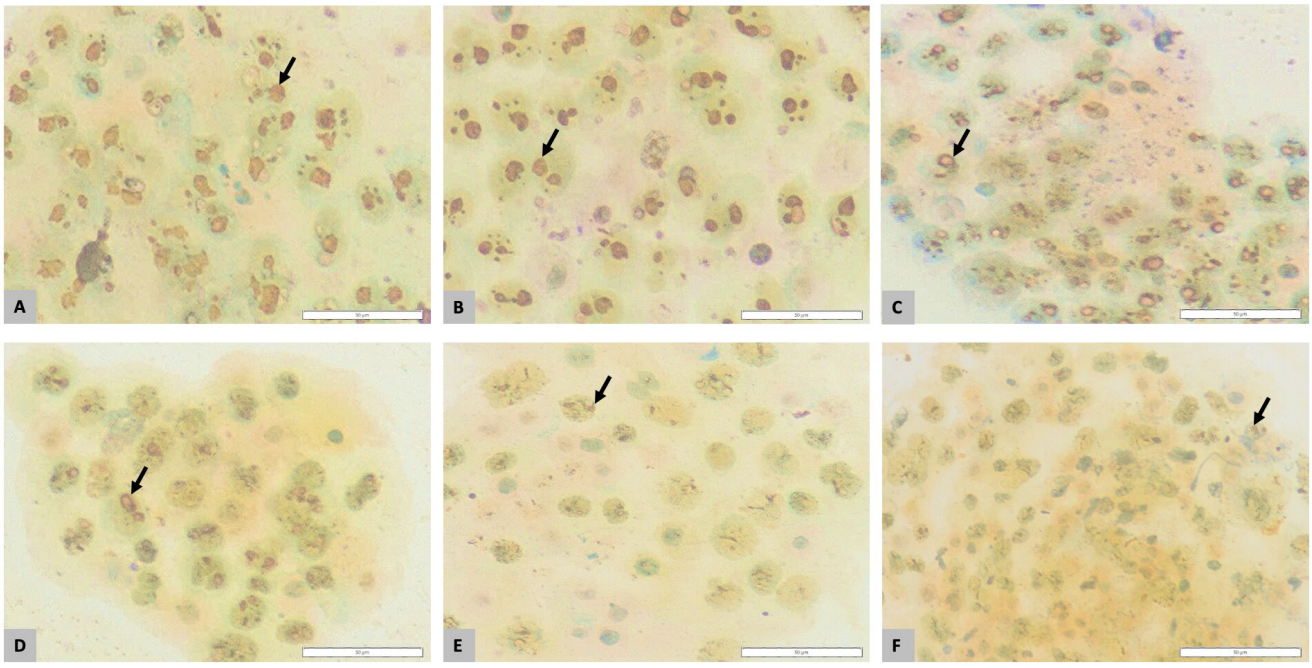

Fig. 6. Image examples of the AgNOR staining cells. A. Control group, B. 5-FU group, C. 100 µg / ml mistletoe group, D. 200 µg/ml mistletoe group, E. 400 µg / ml mistletoe group, F. 800 µg/ml mistletoe group

Table 2. Mean AgNOR number after 24, 48, and 72 hours of incubation

Hours/Groups	Control	5-FU	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml	p
24 hours	3.54 ± 1.26^{a}	2.82 ± 1.06^{b}	2.98 ± 1.16^{ab}	1.74 ± 0.77^{c}	1.74 ± 1.00^{c}	1.80 ± 1.19^{c}	<0.001
48 hours	3.68 ± 1.55^{a}	3.16 ± 1.07^{a}	2.40 ± 0.78^{b}	2.12 ± 1.25^{b}	1.96 ± 0.75^{b}	1.28 ± 0.53^{c}	<0.001
72 hours	1.52 ± 0.64^{a}	2.20 ± 1.08^{bc}	2.26 ± 1.24^{c}	2.20 ± 1.01^{bc}	1.68 ± 0.76^{ab}	1.74 ± 0.85^{abc}	<0.001

p <0.05 was considered statistically significant. Data are expressed as mean ± SD (standard deviation). There is no statistically significant difference between the groups containing the same letter (p> 0.05). 5-FU: 5 Fluorouracil; 100, 200, 400, 800 µg / ml: Mistletoe groups. AgNOR: Argyrophilic nucleolar organizer region.

Table 3. TAA / NA value at the end of 24, 48, and 72 hours of incubation

Hours/Groups	Control	5-FU	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml	p
24 hours	0.11 ± 0.04^{ab}	0.14 ± 0.04^{b}	0.10 ± 0.03^{ac}	0.13 ± 0.06^{ab}	0.08 ± 0.04^{c}	0.10 ± 0.05^{ac}	<0.001
48 hours	0.27 ± 0.15^{a}	0.23 ± 0.05^{a}	0.17 ± 0.08^{b}	0.13 ± 0.12^{bc}	0.09 ± 0.05^{c}	0.09 ± 0.07^{c}	<0.001
72 hours	0.10 ± 0.04^{ac}	0.12 ± 0.04^{ab}	0.13 ± 0.05^{b}	0.10 ± 0.06^{ac}	0.08 ± 0.04^{c}	0.11 ± 0.04^{bc}	<0.001

p <0.05 was considered statistically significant. Data are expressed as mean ± SD (standard deviation). There is no statistically significant difference between the groups containing the same letter (p> 0.05). 5-FU: 5 Fluorouracil; 100, 200, 400, 800 µg / ml: Mistletoe groups. TAA/NA: Total AgNOR area (TAA)/Total nuclear area (NA) ratio.

cubation, the mean AgNOR number was significantly lower in the 5-FU and 200, 400, and 800 µg/ml mistletoe groups compared to the control group (p <0.05). In the data from the 48-hour incubation, this value was significantly lower (p <0.05) in all mistletoe groups compared to the control group. In the data from the 72-hour incubation, this value was significantly higher (p <0.05) in the 5-FU group and the 100 and 200 µg/ml mistletoe groups (Table 2).

When looking at the data from the 24-hour incubation, TAA/NA value was significantly lower in the mistletoe group at 400 µg/ml compared to the control group ($p < 0.05$). In the data from the 48-hour incubation, this value was significantly lower ($p < 0.05$) in all mistletoe groups compared to the control group. In the data from the 72-hour incubation, this value was significantly higher ($p < 0.05$) in the 100 µg/ml mistletoe group (Table 3).

4. Discussion

Cancer is an increasingly common public health problem with an increasing prevalence around the world [18]. Chemotherapy, radiotherapy, and surgery are among the traditional treatments mainly used in cancer. The fact that traditional cancer treatment methods have different side effects, and that they have low effectiveness in some cases, push people to seek different ways to treat this disease [19, 20, 21]. Nowadays, complementary treatment methods are used in the treatment of many diseases, including cancer. Herbal treatments stand out among the complementary treatment methods [22, 23].

In a review about the relationship between nutrition and cancer, it was found that a healthy and balanced diet effectively reduced traditional treatment-related side effects, and a relationship between nutrition and life span was also suggested [21]. It has been observed that the combined use of herbal supplements and chemotherapy, which is one of the complementary cancer treatment methods, increases the success of the treatment [24].

In previous studies, it has been reported that the use of mistletoe as a complementary therapy in addition to traditional cancer treatment reduces general negativities, increases survival time, and shows a synergistic effect with surgery and radiotherapy [5, 6, 25, 26].

While the lectin found in mistletoe shows the most cytotoxic effect on colon cancer cells, depending on time and dose, it has been stated that this effect is low on epidermal cancer cells and has no effect on normal cells [27].

In a study evaluating the effectiveness of the combined use of Trastuzumab, an antitumoral drug, and the mistletoe-derived viscum album extract (VAE) on a human breast cancer cell line, it was reported that the single or combined use of VAE and Trastuzumab reduced proliferation. However, it has been stated that while high doses of VAE provide apoptosis, Trastuzumab does not have such an effect [28]. In another study, it has been shown that the chemical composition of VAE affects in vitro tumor cell death mechanisms and has a potential use in cancer pharmacotherapy research [29]. It is reported that the extract from mistletoe found on apple trees reduces the proliferation of Ewing sarcoma cells and induces apoptosis in a dose-dependent manner [30].

In a study conducted on mice with Ehrlich acid tumor (EAT), it has been shown that this herb has a strong antitumoral effect by regulating granulocyte levels and neutrophil reactions and increasing the number of white blood cells [31]. In another a similar study, it was reported that mistletoe extract developed a protective effect in EAT-injected female Swiss mice; oxidative changes in EAT cells caused a decrease in catalase activity and an increase in xanthine oxidase and peroxidase activities [32].

It has been reported in the literature that viscum album extract (VAE) (50-400 µg/ml) inhibits the proliferation of SK-Hep1 cells in a dose-dependent manner, and also has an anticarcinogenic effect by arrest the cell cycle at the G_1 stage [33]. In another study, it was concluded that VAE treatment arrests the cell cycle at the G_2/M stage [28].

Mitochondria play an important role in the regulation of caspase-3 and -9 activity. With the decrease of mitochondrial membrane potential, cytochrome C becomes free and apoptosis begins with caspase-3 and -9 activity [34]. It is stated that mistletoe lectin II causes breaks in DNA according to time and dose and as a result, causes DNA to take the ladder image and activation of caspase-3, -8 and -9 in U937 cells. In addition, it has been reported that both PARP (poly-ADP-ribose polymerase) and PKC-δ (protein kinase C-δ) decompose in U937 cells to which mistletoe lectin II is applied, as a result of the catalytic activation of caspases [35, 36].

It has been reported that mistletoe has a proliferation-reducing and caspase-mediated apoptosis effect on the most common childhood cancer, and on two different cell lines (Daoy and ONS-76) of medulloblastoma [37].

One of the methods used to evaluate the proliferation status of cancer cells is the AgNORs method. In a study of mice with Ehrlich's ascitic carcinoma (EAC), it was concluded that the average AgNOR count and TAA/NA value found in the tissues of the group treated with curcumin (50 mg/kg) were low compared to the positive control group [38]. In another study, it was reported that the TAA/NA value and AgNOR number decreased in the quercetin-treated groups compared to the untreated group [39]. In other similar studies, it was reported that the AgNOR number in the treatment groups decreased [14, 40].

Mistletoe has an effect mechanism against cancer by affecting different signaling pathways; this was reported in reviews where many different studies, conducted in vitro and in vivo, were examined. There are also studies showing that it is effective on structures such as membrane receptors, enzymes, ion channels, and carrier proteins. As a result of the review, it has been reported that mistletoe inhibits tumor formation and growth, and positive results have been obtained [41, 42].

According to the results of the annexin V & dead cell test of our study, it was observed that the proliferation of the mistletoe- and 5-FU-applied EAT cells decreased and apoptosis was stimulated. In DNA content profile analysis, it was seen that 5-FU administration arrested the cell cycle at the G_0/G_1 stage. Mistletoe application was observed to arrest the cell cycle at the S stage and the G_2/M stage. According to mitochondrial membrane potential evaluations, it was observed that mistletoe application increased the depolarization of EAT cells and this effect was higher espe-

cially in the 800 µg/ml mistletoe group. In the evaluation with AgNOR staining, it was concluded that the application of 5-FU and mistletoe decreased the proliferation of EAT cells.

5. Conclusions

5-FU showed an antiproliferative effect by inducing apoptosis and arresting the cell cycle at the G0/G1 stage. It was observed that mistletoe increased apoptosis, arrested the cell cycle at the S and G2/M stages, and also exerted an antiproliferative effect by increasing apoptosis through depolarization of cells. It was seen that the results obtained and the results of other studies in the literature support each other. Previous studies and current study results strengthen the opinion that mistletoe can be an effective agent against cancer.

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Authors' Contribution

All authors contributed to the study. Performed experiments and first draft of the manuscript (Şükrü Ateş and Harun Ülger); extraction of mistletoe fruit and total phenolic and flavonid content analysis (Gökçe Şeker Karatoprak); study conception, design and laboratory facilities (Şükrü Ateş, Özge Al, Sümeyye Uçar and Mustafa Taştan); AgNOR staining (Şükrü Ateş, Şerife Alpa and Adem Tokpinar); intellectual support and final manuscript (Seher Yilmaz and Ammad Ahmad Farooqi). All authors read and approved the final manuscript.

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Conflict of Interest

The authors have no potential conflicts of interest to declare.

Ethics Approval

Approval of the Local Ethical Committee for Animal Experiments at the Erciyes University was obtained, decision no. 19/053.

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