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Investigation of the effect of yarrow (*Achillea millefolium*) on Ehrlich ascites tumor

Original Study

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Abstract

Introduction. One of the most important health problems today is cancer. The aim of this study was to investigate the in vitro effect of yarrow (Y) with known anticarcinogenic effect on Ehrlich ascites tumor (EAT).
Materials and Methods. The above-ground part (300 g) of Y was macerated with water and extracted three times for 24 hours at 37°C in a shaking water bath. In the study, EAT cells were divided into control, DMSO group 5-FU, 50, 100, 200, 400 and 800 µg/ml YP groups.
Results. At the end of the hour, it was observed that total apoptosis increased significantly in Y groups (especially 50 µg/ml) compared to the control group ($p<0.05$). It was observed that Y slowed the division of EAT cells (especially 800 µg/ml) by stopping the cell cycle at the G0/G1 stage. It was concluded that Y (especially at high doses) triggered apoptosis by significantly increasing the percentage of total depolarized cells ($p<0.001$) in all three time periods.
Conclusions. The results obtained showed that Y extract may have an antitumoral effect on EAT cells. It is thought that this study will contribute to studies on cancer treatment.

Keywords

animal • cell • yarrow • EAT • apoptosis

1. Introduction

Natural products obtained from plants provide important clues in the drug treatment process. Drug discovery is primarily about researching studies of substances traditionally used to treat a disease. What is known today about plants and their applications in medicinal treatments is passed orally from one generation to another [1]. Documenting such information can help preserve and facilitate future research into medicinal plant safety and validity. Therefore, research is needed to better understand the characteristics, safety, and productivity of such plants and at the same time to prevent changes in the knowledge of medicinal plants during the intergenerational transition [2].

The relationship between the properties of plants (aroma, color, odor, and appetite) with psychology, obesity, and metabolism is on the agenda as well. This has increased the tendency to use herbal products for therapeutic purposes, and drugs with natural active ingredients have begun to be preferred. Throughout history, humankind has tried to find a solution to many microbial, metabolic, and psychological health problems with plants. For this reason, the methods of treating some diseases with herbs are based on deep experience and have yielded very successful results, so the use of herbal products in treatment continues today [3].

The genus *Achillea*, belonging to the *Asteraceae* family, includes more than 130 perennial plant species from the Asian continent to Europe. *Achillea millefolium* L. (yarrow or milfoil, the best known and most common species) has been listed among the most used plant species in both folk medicine and traditional medicine for over 3,000 years [4]. It shows various pharmacological properties, such as analgesic, anti-inflammatory, antidiabetic, antioxidant, antiseptic, and antifungal [5].

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Yarrow (Y) is a plant that has been used in many countries of the world for hundreds of years; its scientific name is *Achillea millefolium*. Y has flavonoid and antioxidant properties and is used to treat many diseases [6].

It is known that Y has an antitumoral effect on cancer. It has effects on various types of tumor cells, such as breast epithelial adenocarcinoma, lung tumor cells, leukemia, skin epidermoid carcinoma, hepatoma, and potentially further anticancer activity [5].

The antitumoral and antioxidant effects of herbal extracts have been tested on many cancer models, one of which is the Ehrlich ascites tumor (EAT) model. EAT first appeared as spontaneous mammary adenocarcinoma in a female mouse and was transformed into experimental tumors after subcutaneous transplantation from mouse to mouse [7].

This study aimed to investigate the in vitro effect of Y, which has a known anticarcinogenic effect on Ehrlich ascites tumor (EAT).

2. Materials and Methods

2.1. Preparation of EAT cells

The cells to be used to determine the in vitro effect of the yarrow extract on EAT cells were obtained from our warehouse at –80^{0}C. After the frozen cells were thawed in the incubator, set at 37^{0}C, the supernatant was taken into the centrifuge tube and centrifuged at 3000 min/revolution for 5 min, and the standard medium was added to the cells and the tube was vortexed. Viability examination of the cells was made on the basis of trypan blue staining. For this, 100 µl was taken and 400 µl DPBS was added to the liquid, which was placed in an empty Eppendorf tube. The mixture obtained was homogenized with a pipette. 100 µl of the mixture was taken into another Eppendorf tube and 100 µl of 10% trypan blue was added and mixed. 50 µl of the final mixture was placed in the sample chambers of the Thoma slide, covered with coverslip, and examined under a light microscope, first with a 10X power and then a 40X power objective lens, and then stained and unstained cells were counted and noted. Blue stained cells were considered dead, bright white cells were considered alive. The same procedure was followed to determine the total number of cells in one ml according to the Thoma slide counting formula (Figure 1).

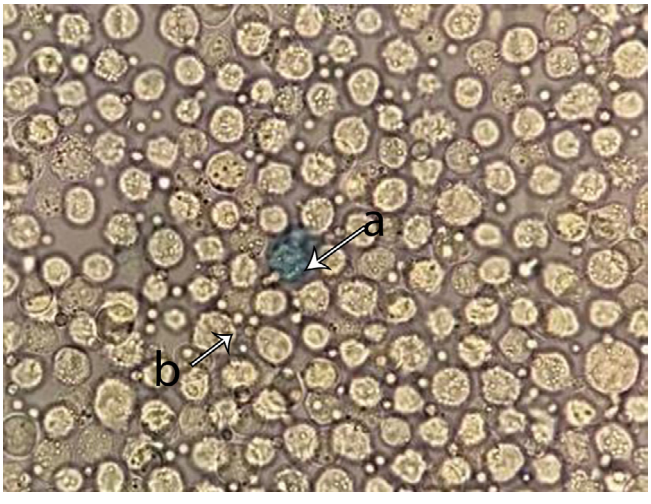

Fig. 1. EAT cells view under light microscope a:Dead EAT cell b:Live EAT cell (X40)

Total number of cells in 1 ml= the number of cells in the whole area x dilution rate x 10,000.

2.2. Preparation of yarrow extract

The above-ground part of the yarrow plant to be used in the study was collected from Erciyes University İncesu Campus. Plant samples are preserved in Erciyes University, Faculty of Pharmacy, Department of Pharmacognosy.

Vegetal material (300 g) was extracted with 70% methanol (MeOH) and ethanol (EtOH), three times for 24 hours at 37°C in a shaking water bath. The extracts obtained were concentrated in a rotavapor (37–38°C) (Heidolph, Laborota 4000, Germany) under vacuum. Solvent-removed extracts were frozen at –80°C after dispersing in water and then lyophilized (Labconco Freeze Dryer, Kansas City, MO, USA). All extracts stored at –18°C until analysis.

2.3. Analysis of EAT Cells

24-well culture dishes were used to determine the in vitro effect of the lyophilized powder extract of yarrow on EAT cells. After 24, 48, and 72 hours of culture, cells were subjected to three different analyses, as Annexin V, Mitopotential, and cell cycle. The doses of mercury to be applied to EAT cells were determined as 50-100-200-400-800 µg/ml. DMSO group was added as the yarrow extract was dissolved in distilled water and 0.1% DMSO mixture. A control group with only cancer cells and medium was formed. 5-Fluorouracil, one of the commonly used chemotherapeutic agents, was also administered at a dose of 60µM. Three repetitions were done for each group. To each well, EAT cells were seeded: 50,000 for Annexin V, 200,000 for mitopotential assay, and 1,000,000 for cell cycle. Each well was pipetted separately with a sterile disposable pipette tip to mix the cells and the medium. After the planting process was completed, the culture containers were removed to the incubator and kept in an environment with 5% CO_2 at 37°C. In the meantime, one 1.5 ml standard tube for each well was labeled according to the groups.

2.4. Annexin V Test

Muse Cell Analyzer (Millipore, USA) device and Muse Annexin V and Dead Cell kit compatible with the device were used to perform the test. The Muse Annexin V and Dead Cell kit used An-

nexin V to detect PS in the outer membrane of apoptotic cells. With this test, the percentages of cells in early apoptotic phase, late apoptotic phase, and total apoptotic cells were determined.

The experiment was planned to cover three different periods of 24, 48, and 72 hours. Sterile disposable culture dishes with 24 wells were used for culturing the cells. The names of the groups were written on the upper sides of the lids of the culture containers with acetate pen to determine which group will be placed where. 50,000 EAT cells were placed in each well with the medium (Figure 2).

The wells of the culture dishes removed from the incubator 24 hours after the seeding were transferred to their Eppendorf tubes by means of a pipette and sterile pipette tips. In the meantime, the Annexin V kit was taken out of the refrigerator and brought to room temperature.

While the wells were emptied, the culture dish was held at an angle of approximately 45 degrees and the liquid was pipetted off in case the cells collapse. Tubes were centrifuged at room temperature at 300 g for five minutes in a fixed angle rotor. After centrifugation, after the supernatant on the tubes was removed with the help of a micropipette, one ml sterile DPBS was added to each tube. After adding DPBS, the vortexed tubes were again centrifuged in a fixed angle rotor at 300 g for five minutes at room temperature. After the supernatant on the tubes was removed, 100 µl 1% fetal bovine serum and 100 µl Annexin V kit liquid were added to each tube. The tubes were vortexed and incubated for 20 minutes at 24^{0}C in the dark.

During this waiting period, the Muse Cell Analyzer device was pre-washed with standard solutions and the device was made ready for sample reading. At the end of the twentieth minute, the tubes were placed one by one into the sample chamber of the device, allowing the device to read the results of apoptosis. The reading process was started from the control group and while the first sample was being read, the area where the cells were concentrated was detected on the device's screen and the door was adjusted. After selecting the dilution rate, the other samples continued to be read. At the end of each group, the device was quickly washed. After all results were saved in the memory of the device, they were taken with the help of a portable disk. The same procedures were repeated after 48 and 72 hours and the results were recorded.

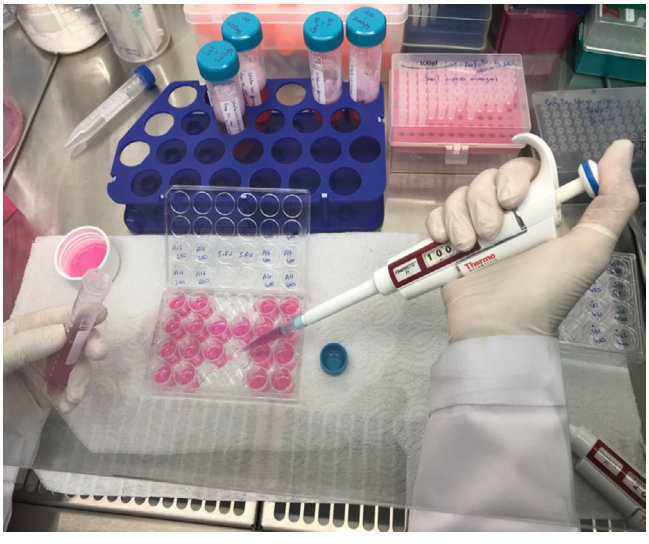

Fig. 2. Addition of cells and media to sterile culture dishes under safety cabinet

2.5. Cell Cycle Test

The Muse cell cycle kit used propidium iodide-based staining of DNA content to distinguish and measure the percentage of cells in all cell cycle phases (G0/G1, S and G2/M).

The Muse Cell Analyzer device and the compatible Muse Cell Cycle kit were used to perform the test. With this test, easy and fast quantitative measurements of the cell percentage in the G0/G1, S and G2/M phases of the cell cycle were provided in the Muse Cell Analyzer. One day before starting the experiment, necessary media and solutions were prepared. 70% ethyl alcohol, 100% absolute alcohol, and distilled water were required for the test; After 70 ml of alcohol was prepared according to the ratio of 30 ml of distilled water, it was kept at -20°C in a sealed bottle.

While the wells were emptied, the culture dish was held at an angle of approximately 45 degrees and the liquid was pipetted off in case the cells collapse. After the caps of the tubes were closed, they were centrifuged at room temperature 300 g for 5 minutes in a fixed angle rotor. After centrifugation, after the supernatant on the tubes was removed with the help of a micropipette, one ml sterile DPBS was added to each tube. The tubes were vortexed at medium speed for 3-5 seconds after DPBS was added, and then centrifuged at 300 g for 5 minutes at room temperature in a fixed angle rotor. The supernatant on the tubes was removed with the help of a micropipette and 50 µl DPBS was added to each of the tubes, then low speed vortex was taken and while mixing was continued, one ml of 70% ethanol was slowly added to it at -20°C, and the caps of the tubes were closed and vortexed. All of the tubes were lined up in a support and kept at -20°C overnight.

After the tubes taken out of the freezer at the end of one night were vortexed, they were taken to the new Eppendorf tubes with 200 µl each, and their caps were closed and centrifuged at 300 g for 5 minutes in a fixed angle rotor. After the supernatant on the tubes was removed with the help of a micropipette, 250 µl DPBS was added to each tube and the tubes were vortexed for 3-5 seconds. Then, the tubes were centrifuged at 300g in a fixed angle rotor for 5 minutes, after the supernatant was removed with the help of a micropipette, 200 µl Muse Cell Cycle kit was added to each of the tubes and the tubes were

vortexed at medium speed for 3-5 seconds. It was kept for 30 minutes at room temperature in a dark drawer. During this waiting period, the Muse Cell Analyzer device was turned on and the device was made ready for sample reading by pre-washing the device with standard solutions.

At the end of the 30th minute, the tubes were placed one by one into the sample chamber of the device, allowing the device to read the cell cycle results. The reading process was initiated from the control group and while the first sample was being read, the area where the cells are concentrated was detected on the device's screen and the door adjustment was made and the other samples continued to be read (Figure 3).

At the end of each group, the device was quickly washed. After all results were saved in the memory of the device, they were taken with the help of a portable disk. The same procedures were repeated after the 48 and 72 hour periods and the results were recorded. After the procedures, the Muse Cell Analyzer device was turned off after the necessary washing.

2.6. Mitochondrial Membrane Potential Assay

As a result of the test of mitochondrial potential and cellular plasma membrane permeability, the percentage of depolarized, viable, depolarized/dead, and dead cells and concentrations (cells/mL) for total, viable, depolarized, depolarized/dead, and dead cells were obtained. The Muse mitochondrial potential test used a cell marker (7-aminoactinomycin) as an indicator of cell death, a cationic, lipophilic dye, and the MitoPotential dye, to detect changes in mitochondrial membrane potential. The media required for the experiment was prepared one day before starting the study. On the day of the experiment, the standard mitochondrial potential solution required for the experiment was prepared. For this purpose, 1 µl of mitopotential dye and 1999 µl of mitopotential assay buffer liquid were added for each tube. The solution obtained was kept in the dark at room temperature until the moment of use.

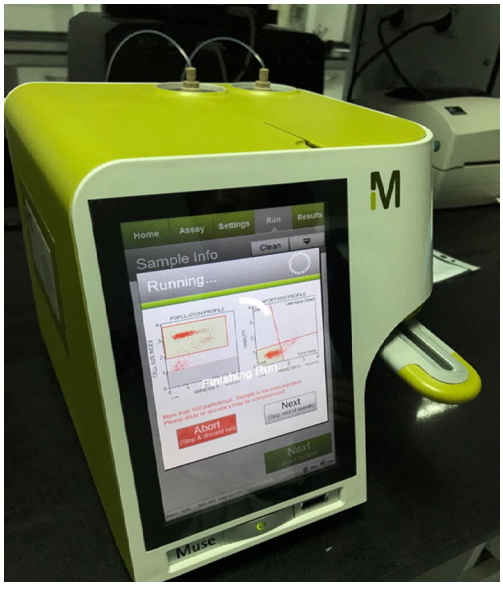

Fig. 3. Reading the results on the Muse cell analyzer device

The wells of the culture dishes removed from the incubator 24 hours after planting were transferred to their Eppendorf tubes by means of a pipette and sterile pipette tips. While the wells were emptied, the culture dish was held at an angle of about 45 degrees and the liquid was thoroughly pipetted off in case the cells collapse. After the tubes were tightly closed, they were centrifuged in a fixed angle rotor at 1200 rpm for 5 minutes at room temperature. After centrifugation, after the supernatant on the tubes was removed with the help of a micropipette, one ml sterile DPBS was added to each tube. The tubes were vortexed at medium speed for 3-5 seconds after DPBS was added, and then centrifuged again at 1200 rpm at room temperature for 5 minutes in a fixed angle rotor. After the supernatant on the tubes was removed with the help of a micropipette, one ml of mitopotential assay buffer was added to each of the tubes, then the tubes were vortexed and 100 µl of each tube was taken into the new labeled Eppendorf tubes and 95 µl of a pre-prepared standard mitochondrial potential solution was added to them and the tubes were vortexed at 37°C. They were then kept in an incubator for 20 minutes. During this waiting period, the Muse Cell Analyzer device was turned on and the device was made ready for sample reading by pre-washing the device with standard solutions.

At the end of the twentieth minute, the tubes were removed from the incubator, their caps were opened, 5 µl of 7-AAD reagent was added to each tube, and the tubes were capped and vortexed. Then, the tubes were kept at room temperature in the dark for five minutes, and then the reading process was started on the device. The reading process was started from the control group and while the first sample was being read, the area where the cells are concentrated was detected on the device's screen, the door was adjusted, and the other samples continued to be read. At the end of each group, the device was quickly washed. After all results were saved in the memory of the device, they were transfered with the help of a portable disk. The same procedures were repeated after the 48 and 72 hour periods and the results were recorded. After the procedures, the Muse Cell Analyzer device was turned off after the necessary washing.

2.7. Statistical analysis

The distribution of the data was evaluated by histogram, Q-Q plot, and Shapiro Wilks test statistics. One-way analysis of variance was used for the average comparison of more than two independent groups in normally distributed data.

Variance homogeneity was evaluated by Levene Test statistics. Comparison of more than two dependent groups was tested by two-way analysis of variance. Multiple comparison tests of

variables between groups were evaluated with Tukey, Bonferroni, and Tamhane test statistics. Significance level was accepted as p <0.05. The analysis of the data in the study was performed in TURCOSA statistics software (www.turcosa.com.tr).

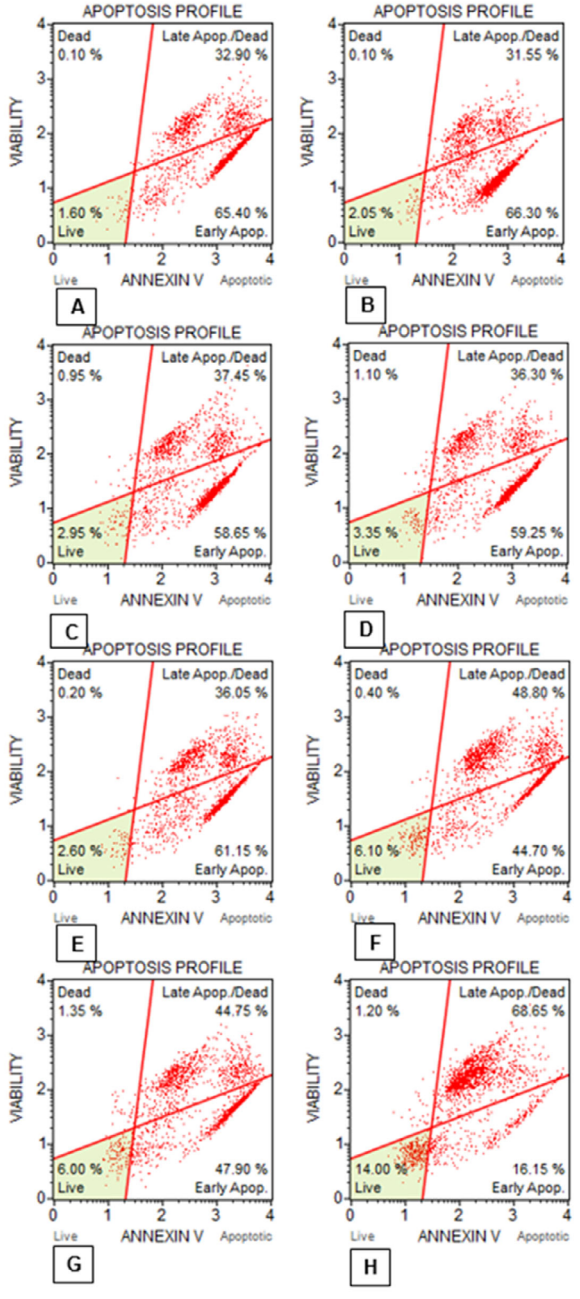

Fig. 4. Data graphics obtained from the Muse Cell Analyzer, showing the apoptosis rates of EAT cells according to the groups at the end of 24 hours of incubation. A. Control group, B. 5-FU group, C. DMSO group, D. 50 µg/ml YP group, E. 100 µg/ml YP group, F. 200 µg/ml YP group, G. 400 µg/ml YP group, H. 800 µg/ml YP group

3. Results

3.1. Annexin V Results

In this test, the percentage rates of cells in early apoptosis, late apoptosis, and total apoptosis were compared statistically between the groups for 24, 48, and 72 hours, and the results were noted. The raw data graphics of the results are shown in Figure 4.

3.2. Early and Late Apoptosis Results

At the end of all three of the 24, 48, and 72 hour periods, it was noted that early apoptosis significantly decreased in the high-dose Y groups compared to the control group (p <0.05). At the end of 24 and 48 hour periods, late apoptosis occurred especially in 800 µg/ml Y group; It was noted that it increased significantly in the 200, 400, and 800 µg/ml Y groups compared to the control group in the 72 hour period (p <0.05). (Table 1 and Table 2).

3.3. Total Apoptosis

While there was no significant difference between the groups in terms of total apoptosis after 72 hours of culture, a significant increase was observed in the ratio of total apoptosis at the end of 48 hours of culture, especially at the dose of 50 µg/ml, compared to the control group (p <0.05) (Table 3 and Figure 5).

3.4. Cell Cycle Results

Within this test, the percentages (%) of cells in the G0/G1, S, and G2/M total phases between groups were compared at the end of 24, 48, and 72 hours of culture. The raw data graphics of the results are shown in Figure 8, Figure 9, Figure 10.

3.5. G2/M Phase Results

When the results of cell cycle analysis were examined, no statistically significant difference was found between the groups at the end of 24 hours of culture in the G2/M phase (p<0.05). On the other hand, at the end of 72 hours of culture, it was seen that

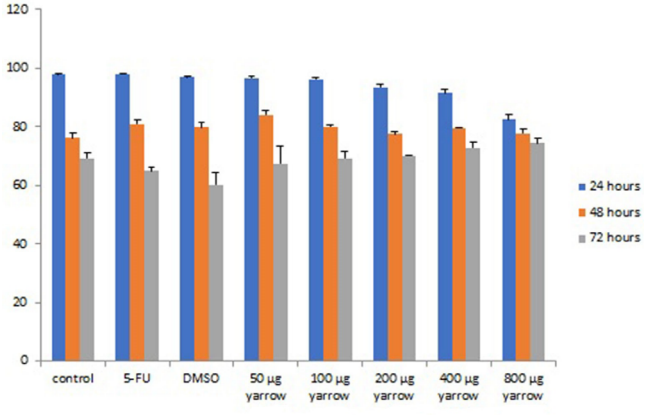

Fig. 5. Percentage values of total apoptosis according to the culture time of the groups

Table 1. Expression of early apoptosis values by groups and time

Early apoptosis	Incubation times		
Groups	24 hours	48 hours	72 hours
Control	65.40±0.20^{a}	45.83±0.42^{ab}	32.75±33.80^{a}
5 FU	67.50±1.37^{a}	53.50±4.47^{a}	27.78±1.02^{ab}
DMSO	63.35±2.55^{a}	41.31±3.54^{ab}	22.83±6.29^{abc}
50 µg/mL Y	61.01±2.10^{ab}	49.15±2.05^{ad}	29.63±5.17^{a}
100 µg/mL Y	60.43±1.03^{ab}	44.13±5.41^{ae}	28.40±1.40^{ab}
200 µg/mL Y	49.05±5.36^{b}	35.41±3.67^{bce}	17.55±3.74^{bc}
400 µg/mL Y	49.13±2.22^{b}	40.76±1.71^{bde}	17.60±3.03^{bc}
800 µg/mL Y	21.80±11.87^{c}	27.55±8.80^{c}	14,60±4,32^{c}
p value between groups	<0.001	<0.001	<0,001

Similar letters show statistical similarity between groups and different letters show statistical difference between groups (p<0.05).

Table 2. Expression of late apoptosis values according to groups and time

Late apoptosis	Incubation times		
Groups	24 hours	48 hours	72 hours
Control	32.40±0.44^{ab}	30.46±2.71^{ab}	36.41±6.22^{a}
5 FU	30.46±1.50^{a}	27.5±2.32^{a}	37.13±1.53^{a}
DMSO	33.48±2.17^{ab}	38.61±6.46^{abc}	37.48±1.72^{a}
50 µg/mL	35.76±1.54^{ab}	35.00±2.08^{ab}	37.86±5.73^{a}
100 µg/mL	35.80±0.66^{ab}	35.86±4.75^{ab}	40.95±2.95^{a}
200 µg/mL	44.50±4.18^{b}	42.26±2.78^{bc}	52.66±3.90^{b}
400 µg/mL	42.40±2.06^{ab}	38.91±1.25^{abc}	55.33±3.44^{b}
800 µg/mL	60.76±11.27^{c}	50.20±7.34^{c}	59.88±1.58^{b}
p value between groups	<0.001	<0.001	<0.001

Similar letters show statistical similarity between groups and different letters show statistical difference between groups (p <0.05).

the 400 and 800 µg/ml Y groups significantly decreased the cell ratio in the G2/M phase compared to the control group (p<0.05), and it was concluded that the Y extract slowed the mitosis of EAT cells (Table 4 and Figure 11).

3.6. Mitotic Activity Results

In this test, living/dead cells, and percentages of depolarized viable, depolarized dead, and total depolarized cells were compared statistically between groups for 24, 48, and 72 hours and the results were noted. Raw data graphics taken from the Muse Cell Analyzer device were taken as basis for the generation of the results. The graphics created by the device were followed in four sections formed by the intersection of the horizontal and vertical axes. The cell populations formed by the markers included in the test were observed in these compartments (depolarized dead cells in the upper left, dead cells in the upper right, depolarized live cells in the lower left, live cells in the lower right). The raw data graphics of the results are shown in Figures 12, 13.

Table 3. Expression of total apoptosis values according to groups and time

Total Apoptosis	Incubation times		
Groups	24 hours	48 hours	72 hours
Control	97.80±0.52^{a}	76.30±3.11^{a}	69.16±3.31^{a}
5 FU	97.96±0.29^{a}	81.05±2.19^{ab}	64.91±2.44^{a}
DMSO	96.83±1.15^{ac}	79.93±2.92^{ab}	60.31±7.24^{a}
50 μg/mL	96,78±0.59^{ac}	84.15±2.23^{b}	67.50±9.92^{a}
100 μg/mL	96.23±0.86^{ac}	80.00±0.98^{ab}	69.35±4.05^{a}
200 μg/mL	93.55±1.2^{bc}	77.68±0.90^{a}	70.21±0.18^{a}
400 μg/mL	91.53±1.84^{b}	79.68±0.45^{ab}	72.93±0.96^{a}
800 μg/mL	82.56±2.67^{d}	77.75±2.77^{a}	74.48±3.06^{a}
p value between groups	<0.001	<0.014	0.072

Similar letters show statistical similarity between groups and different letters show statistical difference between groups (p<0.05).

Table 4. Expression of G2/M phase proportional values according to groups and time

G2/M	Incubation times		
Groups	24 hours	48 hours	72 hours
Control	8.20±1.77^{a}	10.96±1.41^{ab}	17.26±3.90^{a}
5 FU	7.06±1.02^{a}	9.50±2.27^{b}	9.75±2.89^{c}
DMSO	6.20±0.72^{a}	15.13±2.15^{ac}	14.73±1.62^{ac}
50 μg/mL	7.13±0.58^{a}	18.53±1.92^{c}	16.23±2.35^{ac}
100 μg/mL	8.06±1.19^{a}	12.03±1.26^{ab}	11.90±0.91^{ac}
200 μg/mL	8.03±0.11^{a}	13.03±1.28^{ab}	13.96±0.70^{ac}
400 μg/mL	7.63±0.45^{a}	11.93±2.04^{ab}	11.06±0.35^{bc}
800 μg/mL	8.50±1.01^{a}	10.60±2.30^{ab}	11.20±1.40^{bc}
p value between groups	0.158	<0.001	<0.005

Similar letters show statistical similarity between groups and different letters show statistical difference between groups (p <0.05).

3.7. Total Amount of Depolarized Cells

It was concluded that Y (especially at high doses) significantly increased the percentage of total depolarized cells (p <0.001) in 24 and 48 hour time periods, triggering apoptosis (Table 5).

4. Discussion

In traditional medicine, various herbs have been used for medicinal purposes and applied for different treatments [8]. Components of necessary oils of plants have been shown to be effective in alternative and complementary therapies. Some of the well-known ingredients found in the essential oils of medicinal plants are derivatives with anticancer properties used in chemotherapy. Yarrow has also been used for alternative treatments for diabetes, cancer, metabolism, and atherosclerosis [9].

In vitro growth inhibitory activities of 1,10-secoguaianolide isolated from the methanolic extract of Y flower against human tumor (MCF7WT) and human prostate cancer cell line (PC3) were studied. MTT analysis was performed for the cell viability test. The results show that only seco-tanapartholide A shows moderate cell growth inhibitory activity against the human cancer cell line MCF7WT [5].

Cytotoxic effects of *A. millefolium* extract on *Lactuca sativa* root tip meristem cells were investigated. Lettuce seeds were treated with different concentrations of *A. millefolium* extract

(5, 10, 20, 30 mg/mL) for 72 hours to examine root growth, germination percentage, and cellular behavior. The results showed that 30 mg/mL extract reduced seed germination, mitotic index, and root growth, as well as causing chromosome aberrations in *L. sativa* stem cells. Though *A. millefolium* has a beneficial effect as a medicinal plant, it can cause severe damage to cells due to improper handling [10].

De Santanna et al. [11] investigated the genotoxic activity of *A. millefolium* essential oil on the heterozygous diploid strain of *Aspergillus nidulans* (A757//UT448 with green conidia). They reported a significant increase in the number of yellow and white

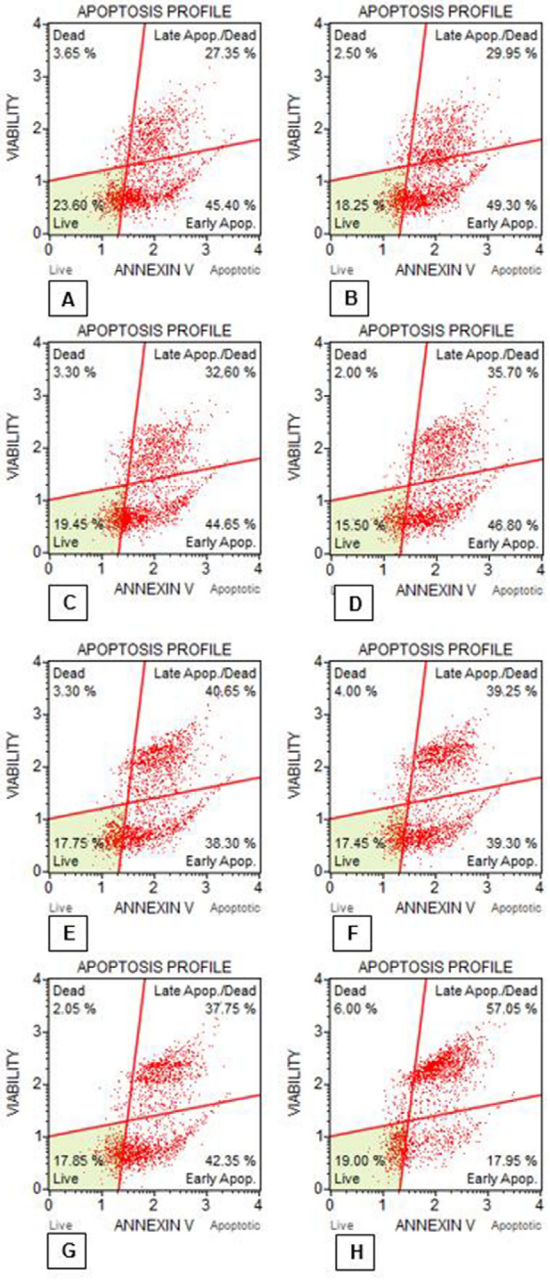

Fig. 6. Data graphics obtained from the Muse Cell Analyzer, showing the apoptosis rates of EAT cells according to the groups at the end of 48 hours incubation. A. Control group, B. 5-FU group, C. DMSO group, D. 50 µg/ml YP group, E. 100 µg/ml YP group, F. 200 µg/ml YP group, G. 400 µg/ml YP group, H. 800 µg/ml YP group

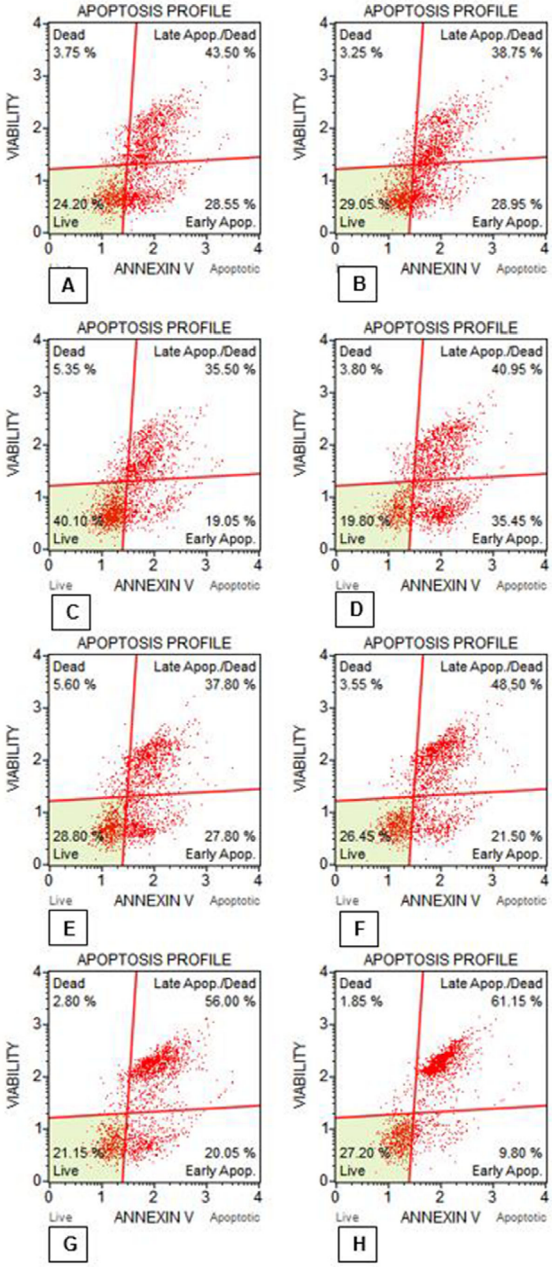

Fig. 7. Data graphics obtained from the Muse Cell Analyzer, showing the apoptosis rates of EAT cells according to the groups at the end of 72 hours of incubation. A. Control group, B. 5-FU group, C. DMSO group, D. 50 µg/ml YP group, E. 100 µg/ml YP group, F. 200 µg/ml YP group, G. 400 µg/ml YP group, H. 800 µg/ml YP group

mitotic recombinants per diploid strain colony after oil treatment with 0.19 and 0.25 µL/mL. The genotoxicity of the oil has been associated with induction of mitotic separation or passage by the oil.

The antitumor potential of castefolium isolated from *A. millefolium* was studied through cell cycle and apoptotic signaling pathways in two MCF-7 sublines, MN1 and MDD2. Both cell lines were found to be susceptible to casticin at concentrations greater than 0.25 µM and were reported to exhibit a similar 50% inhibitory concentration (IC50) of 2 µM. Casticin causes cell growth arrest during the G2/M phase and induces apoptotic death by acting as a tubulin binding agent (TBA). They also showed that it inhibits Cdk1 by inducing p21 and downregulates cyclin A expression [12].

Pharmacological properties of *A. Millefolium* include effects on inflammation, bacteria, dyspepsia, parasites, and infections. More studies are needed to find the exact mechanism and causes behind some of its pharmacological properties; many pharmacological effects of the herb have not yet been scientifically proven. Regarding toxicity, conventional doses of *A. millefolium* appear to be safe, but there are insufficient clinical studies on safe doses of *A. millefolium*. However, one aspect that should be considered in clinical use of *A. millefolium* is its interaction with other drugs. The results of in vitro and preclinical studies should be critically evaluated and integrated into the practical applications of *A. millefolium* [5].

Y is a widely used herb in alternative medicine for different purposes. Y production in our country is around 0.16 tons. Plants

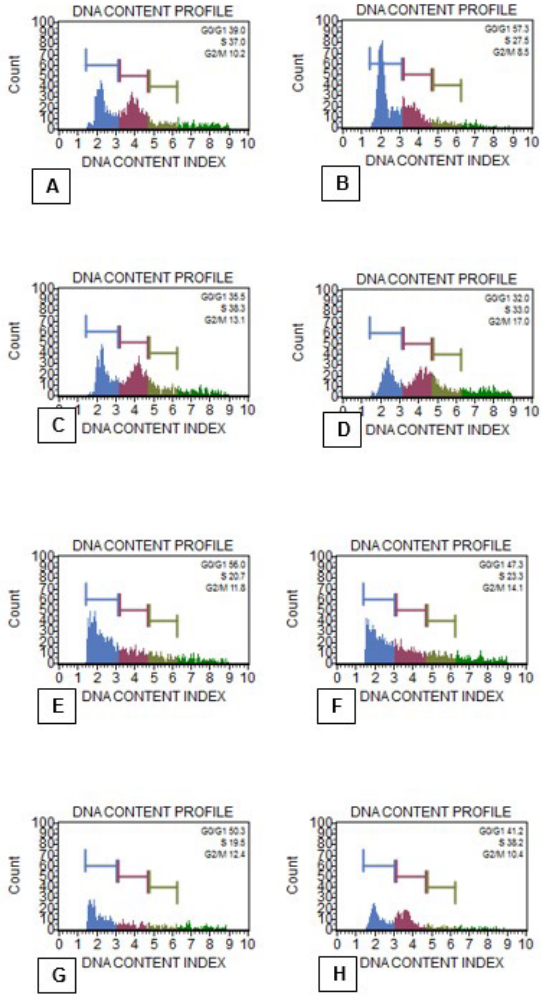

Fig. 8. Data graphs obtained from Muse Cell Analyzer, showing cell cycle rates of EAT cells according to groups at the end of 48 hours of incubation. A. Control group, B. 5-FU group, C. DMSO group, D. 50 µg/ml YP group, E. 100 µg/ml YP group, F. 200 µg/ml YP group, G. 400 µg/ml YP group, H. 800 µg/ml YP group

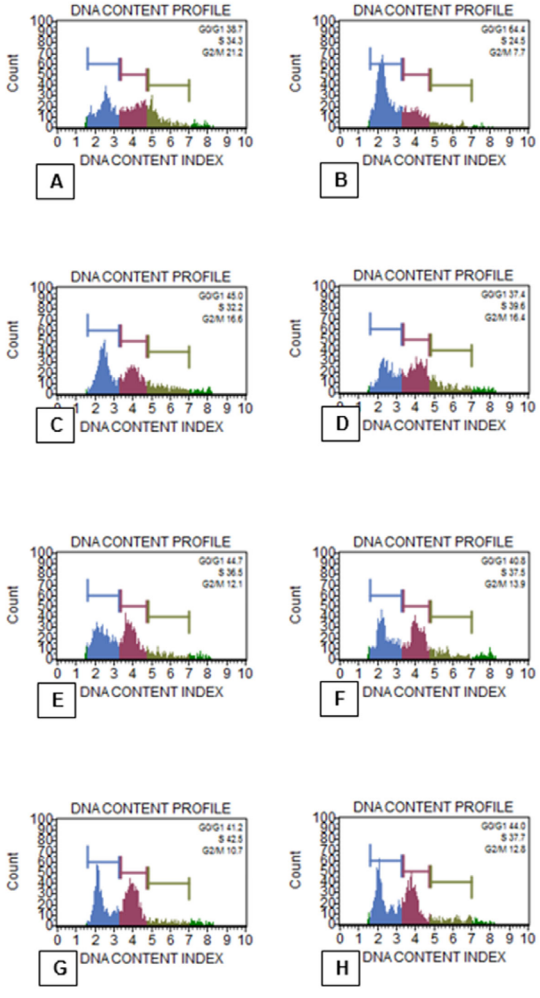

Fig. 9. Data graphs obtained from Muse Cell Analyzer, showing cell cycle rates of EAT cells according to groups at the end of 72 hours of incubation. A. Control group, B. 5-FU group, C. DMSO group, D. 50 µg/ml YP group, E. 100 µg/ml YP group, F. 200 µg/ml YP group, G. 400 µg/ml YP group, H. 800 µg/ml YP group

Table 5. Expressing the proportional values of total depolarized cells by groups and time

Total Amount of Depolarized	Incubation times		
Groups	24 hour	48 hour	72 hour
Control	0.11±0.10^{a}	0.05±0.05^{ab}	0.25±0.10^{a}
5 FU	0.10±0.00^{a}	0.00±0.00^{a}	0.36±0.10^{a}
DMSO	0.28±0.12^{a}	0.03±0.05^{a}	0.25±0.31^{a}
50 μg/mL Y	0.46±0.33^{ab}	0.06±0.02^{ab}	0.28±0.40^{a}
100 μg/mL Y	0.61±0.05^{ab}	0.13±0.02^{ab}	0.40±0.22^{a}
200 μg/mL Y	0.91±0.05^{ab}	0.08±0.02^{ab}	0.33±0.25^{a}
400 μg/mL Y	1.78±0.96^{bc}	0.41±0.23^{b}	0.50±0.44^{a}
800 μg/mL Y	2.96±0.83^{c}	0.86±0.27^{c}	4.80±4.56^{a}
p value between groups	<0.001	<0.001	<0.042

Similar letters show statistical similarity between groups and different letters show statistical difference between groups (p <0.05).

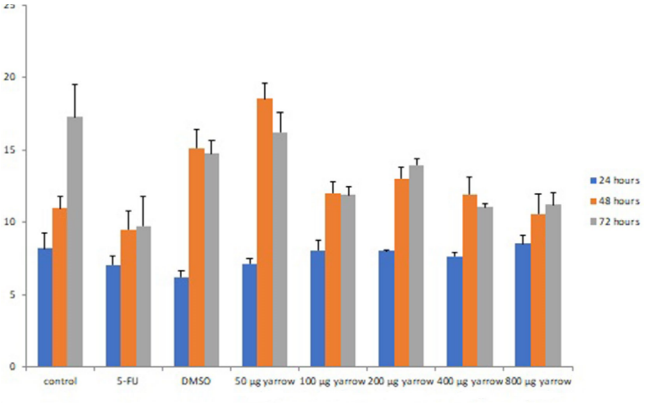

Fig. 10. Percentage values of the G2 / M Stage according to the cultural times of the groups

in the Y group are among the important medicinal plants due to their essential oils, which are rich in kamazulen content. In addition, this plant has flavonoid properties. Flavonoid, one of the compounds in Y, prevents cell growth in its antitumor activity mechanism and causes apoptotic cell death due to the casticin [13].

Besides macronutrients in Y such as free sugars, organic acids, and fatty acids, tocopherols are elements that show very strong antioxidant activity. The main effective compounds against cancer in the yarrow are sesquiterpene lactones [14].

In vitro studies have been carried out on cell lines on Y. Csupor-loffler et al. [15] stated that they had an inhibitory effect on the growth of tumor cells in the MCF-7 cell line, in the human cell line they had done in vitro. Ghavami et al. [16] investigated the effect of ethanol extract of Y on cancer cell lines. These cell lines were AGS, MCF7, SW742, SKLC6, A375, and PLC/yPRF/5. These investigators used the Y extract dissolved in ethanol on these cell lines. The Y extract was dissolved in DMSO and ethanol (50%). The findings determined that it has a stimulating effect on cell death in cancerous cell lines.

The apoptotic effects of *Achillea wilhelmsii* type extracts were investigated in various cancer cell lines. The flavonoid casticin from *Achillea millefolium* has shown a significant effect in cancer treatment. Anticancer effects of various *Achillea* species have been reported in different cancer cell lines. A recent study considered the cytotoxic and proapoptotic effects of methanol and water extracts of *Achillea teretifolia* on DU145 and PC3 PCa cell lines [17]. This study stated that *A. millefolium* extract has antitumor potential by interfering with the progression of the cell cycle and causing apoptosis. It is known that this is due to a high bioactive capacity of chlorogenic acid derivatives, especially in the inhibition of cell growth [18, 19].

In addition, many preclinical and phase I studies have reported that treatment with chlorogenic acid derivatives has beneficial effects in colon, brain and lung cancers, chronic myelogenous leukemia, and breast tumors [20].

In studies investigating the effect of various herbal extracts on cancer cell lines, it has been reported that treatment groups increase the amount of apoptotic cells as a result of Annexin V analysis [21, 22]. In our study, it was found that yarrow extracts increased apoptosis on EAT cells at the end of 72 hours of culture; It was observed that this increase was more pronounced in 800 µg/ml groups (p <0.001).

Many natural phytochemical products can suppress cancerous cell proliferation by halting cell cycle phases [21, 23]. In the G0/G1 phase, cells are stopped at the G1 to S checkpoint transition by suppressing the function of the complex formed by combining cyclin E and cyclin dependent kinase-2, which are the

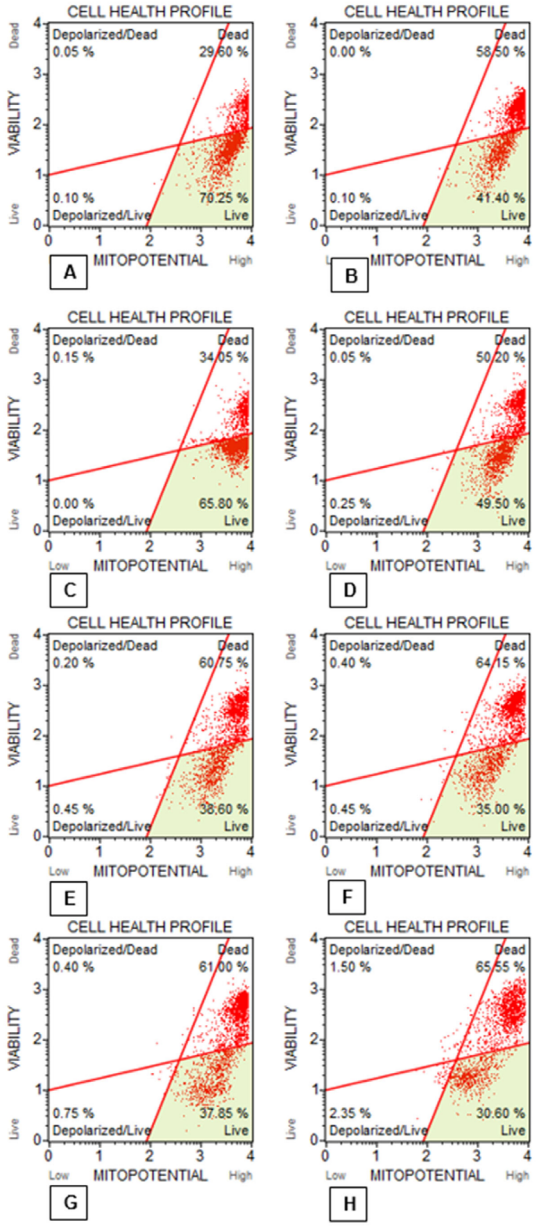

Fig. 11. Data graphics obtained from the Muse Cell Analyzer, showing the mitotic activity rates of EAT cells according to the groups at the end of 24 hours of incubation. A. Control group, B. 5-FU group, C. DMSO group, D. 50 µg/ml YP group, E. 100 µg/ml YP group, F. 200 µg/ml YP group, G. 400 µg/ml YP group, H. 800 µg/ml YP group

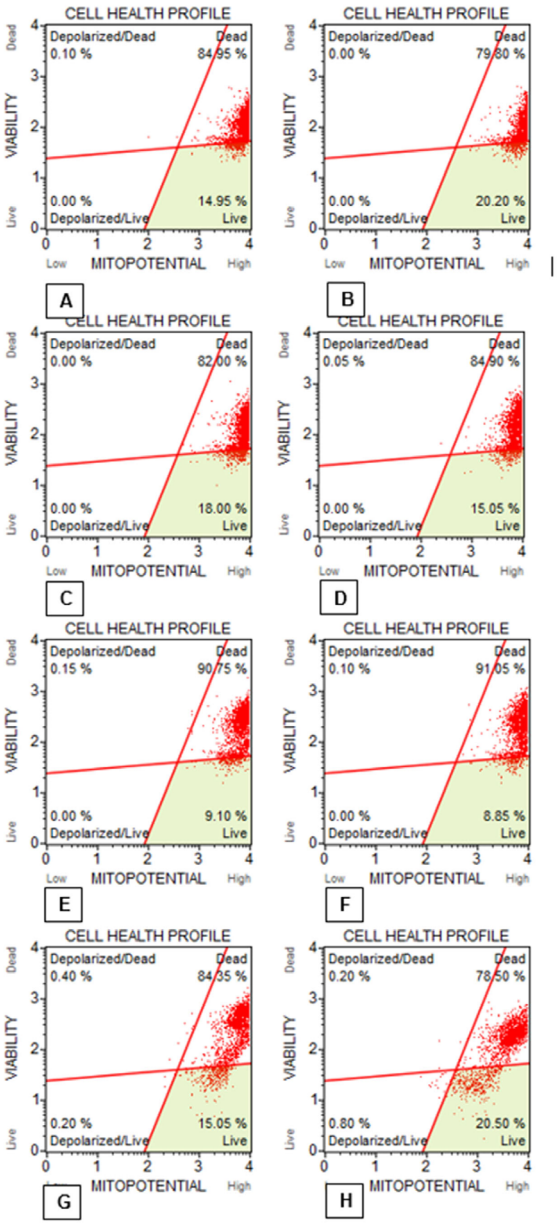

Fig. 12. Data graphics obtained from the Muse Cell Analyzer, showing the mitotic activity rates of EAT cells according to the groups at the end of 48 hours of incubation. A. Control group, B. 5-FU group, C. DMSO group, D. 50 µg/ml YP group, E. 100 µg/ml YP group, F. 200 µg/mlYP group, G. 400 µg/ml YP group, H. 800 µg/ml YP group

main cell cycle regulators, to promote cell transition from the G1 phase to the S control point [24].

Uçar [25], when examining the relationship of goji berry with apoptosis, did not observe a significant difference between the groups after 3 hours of culture, but when compared to the control group, the number of viable cells at the end of 24 and 48 hours showed that it could be caused by inducing apoptosis on goji berry EAT cells. They have stated that it is a general anticarcinogenic substance, preventing the growth of cancerous tissues by inhibiting cell proliferation in liver, stomach, colon, bladder, kidney, and prostate cell lines.

Mao et al. [24], in the study investigating the efficacy of *Lycium barbarum* extract on SW480 and Caco-2 colon cancer cell

lines, reported that the extracts stopped the cell cycle in the G0/G1 phase. In the present study, it was found that all of the yarrow extracts increase the amount of cells in the G0/G1 phase of the cell cycle; It was determined that this increase was more in 800 µg/ml YP groups.

Mitochondrial activity plays a critical role in the regulation of caspase 3 and caspase 9 activation, which are among the main apoptosis parameters. The decrease in mitochondrial membrane potential initiates apoptosis by releasing cytochrome c, which activates caspase 3 and caspase 9 [26].

Lewinska et al. [27], Kim et al. [21], Takeda et al. [26], Lin et al. [28], all reported that the treatment groups decreased the number of viable cells compared to the positive control group and significantly reduced the mitochondrial membrane potential by increasing the number of depolarized cells in their studies investigating the effect of various plant extracts on cancer cell lines. In this study, it was observed that the milky perch reduced the mitochondrial membrane potential, the total percentage of depolarized cells was the highest in the 800 µg/ml Y group, the group with the lowest percentage of live EAT cells and the highest percentage of dead EAT cells was also the group of 800 µg/ml Y.

The fact that these results are similar to the results of studies on apoptosis proves that yarrow extract increases apoptosis of cancer cells by various mechanisms and this effect is clearer in the 800 µg/ml YP group, although this effect is also seen in other groups.

Authors' Contribution

M.N.: research concept and design, supervising the project, final proofreading and approval of the version for publication; **N.İ.**: final proofreading and approval of the version for publication; **A.T.**: carrying out the experiments, data analysis and interpretation; **Ö.A.**: literature review, visualization; **S.U.**: carrying out the experiments, writing—original draft preparation; **M.T.**: carrying out the experiments, acquisition of data; **Ş.A.**: carrying out the experiments, acquisition of data; **S.Y.**: writing—review and editing.

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Conflict of Interest

The authors have no potential conflicts of interest to declare.

Ethics Approval

Since cell culture was performed in this study, no ethics committee approval is required.

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