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Anticancer effect of ethanolic yellow hawthorn extract on chronic myeloid leukemia cells and acute myeloid leukemia cells

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Abstract

Cancer is a disease characterized by abnormal cell growth and invasion and metastasis of these cells to other tissues or organs of the body. Natural products have been used for centuries as drugs or in drug development, especially for the treatment of cancer. Besides, extracting natural products with several bioactive compounds has a promising effect on cancer treatment. In this study, we aimed to investigate the anticancer effect of the ethanolic extract of yellow hawthorn fruits on K562 (Chronic Myeloid Leukemia) and MOLM-13 (Acute Myeloid Leukemia) cell lines. The antiproliferative effect of the ethanolic extract of yellow hawthorn fruits was investigated in time- and dose-dependent manners. The Annexin-V/Propidium Iodide (PI) double staining was used to examine the apoptosis. Furthermore, cell cycle analysis is conducted by PI staining. The cell viability of K562 and MOLM-13 cell lines was significantly reduced by the ethanolic extract of yellow hawthorn fruits with IC50 values of 9144 µg/mL and 3515 µg/mL in 48-hour incubation time, respectively. Moreover, the results showed that the ethanolic extract of yellow hawthorn fruits caused an increased apoptosis by 12.7- and 8.87-fold changes in K562 and MOLM-13 cell lines compared to control groups, respectively. Ethanolic extract of yellow hawthorn fruit has reduced cell proliferation, induced apoptosis and arrested the cell cycle at G0/G1 phase by 71% in MOLM-13 and at G2/M phase by 80.3% and G0/G1 phase by 38.2 % in K562 cells. Further studies should be conducted to elucidate the mechanism of the effect of yellow hawthorn fruit on these cancer cells.

Keywords: yellow hawthorn, ethanolic extract/extraction, natural compounds, cancer, chronic myeloid leukemia, acute myeloid leukemia

1. Introduction

According to the World Health Organization's 2020 data, cancer is the second leading disease in the world, causing approximately 10 million deaths annually (1). Furthermore, it has been stated that the number of deaths it causes in the world will increase to approximately 16.2 million by 2040. Approximately 53,000 people are diagnosed with cancer every day, so it is important to develop novel treatment and diagnosis methods for cancer (2).

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm (MPN) resulting from the clonal proliferation of myeloid progenitor cells in the bone marrow (3). Chronic myeloid leukemia (CML) occurs in 1 or 2 out of 100,000 adults and results from the translocation of the Abelson murine leukemia viral oncogene from chromosome 9 to the BCR on chromosome 22, forming the BCR-ABL1 fusion oncogene. This translocation results in the shortened chromosome 22, known as the Philadelphia (Ph) chromosome (4). Furthermore, this translocation results in the expression of the BCR-ABL oncoprotein, which is a continuously active tyrosine kinase activating downstream signaling pathways such as PI3K/Akt, JAK/Stat5, and Ras/MAPK (5). Moreover, the BCR-ABL oncoprotein ensures the survival, development, inhibition of cell death and self-renewal of cancer cells (6, 7).

Acute myeloid leukemia (AML) is a hematological malignancy formed by genetic alterations in hematopoietic progenitor cells and results in the accumulation of altered cells as myeloid blasts (8, 9). In other words, AML is an aggressive type of cancer that occurs from the proliferation of clonal hematopoietic cells (10, 11). Acute myeloid leukemia (AML), although it can develop at any age, is most commonly diagnosed in individuals over the age of 55, with a median age at diagnosis of 68 years. (12). AML results from various mutations and chromosomal changes (13). In AML, mutations occur most notably in the FMS-like tyrosine kinase 3 (FLT3) (37%), NPM1 (29%), DNMT3A (23%) and N/KRAS (10%) genes (14). FLT3 encodes the receptor tyrosine kinase (RTK) in immature hematopoietic cells. When these immature hematopoietic cells become mature, the FLT3 gene expression is suppressed and tyrosine residues in the FLT3 undergo autophosphorylation and downstream signaling pathways such

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as PI3K/Akt and MAPK are activated and thus abnormal proliferation of cancer cells occurs (15).

Treatment of cancer with natural compounds is an effective method as natural compounds have anticancer effects which may arise from either whole extract of natural compounds or isolated phytochemicals such as, polyphenols, polysaccharides, terpenoids (16). Furthermore, natural products have lower toxicity compared to conventional chemotherapeutics and may decrease the side effects caused by chemotherapeutic drugs. Moreover, these compounds often demonstrate low cytotoxicity on healthy cells (17).

Hawthorn is a plant that grows on thorny shrubs and small trees belonging to the Rosaceae family of the Crataegus genus. It has approximately 280 species and grows in the temperate regions of Europe, East Asia, North America, and 21 species are found in Turkey (18, 19). It has been consumed worldwide for centuries as food, dietary supplements, as well as medicine in traditional treatment methods (20). Its berries, leaves, and flowers have been used for medical purposes for many years due to their antibacterial, anticancer, anti-inflammatory, and antioxidant properties and this plant has been used in the treatment of many diseases, such as cancer, due to its phytochemicals, remarkable effects, and safety profile (21). Hawthorn fruit is rich in various bioactive ingredients such as flavonoids, terpenoids, polyphenols, polysaccharides, and several organic acids (22). Phytochemicals are naturally occurring compounds in plants, fruits, etc. and can suppress the tumor growth, reduce the risk of cancer development by targeting the cellular mechanisms that are altered in cancer progression (23). It is reported that the phenolic extract of Hawthorn (Crataegus pubescens) fruit demonstrates hawthorn has high levels of carbohydrates, terpenes, phenols, and flavonoids (24). Furthermore, it is indicated that the fruits of hawthorn have major phytochemical content rather than its leaves (25). Studies have demonstrated that hawthorn fruit reduces cell proliferation, causes cell cycle arrest, particularly in the G2/M and S phases, and induces apoptotic cell death (26, 27).

Therefore, this research article aimed to investigate the anticancer effect of the extract of yellow hawthorn fruit on K562 and MOLM-13 cells, as no studies have been reported for these cell lines. In the present study, we report the anticancer activities of the ethanolic extract of yellow hawthorn fruit against K562 and MOLM-13 cell lines. Although there is a need for further investigation, our results suggest that the extract used in this study causes a decrease in cellular proliferation, induces apoptosis and causes cell cycle arrest.

2. Materials and methods

2.1. Materials

Soxhlet apparatus, condenser, and absolute ethanol were purchased from ISOLAB. The heating mantle was purchased from Weightlab. Cell lines were obtained from the German National Resource Center for Biological Material (DSMZ). RPMI 1640 was purchased from Serox. The stock solution of ethanolic yellow hawthorn extract is prepared as 250 mg of ethanolic extract and dissolved in 1 mL of RPMI medium for cell culture purposes and stored at -20°C.

2.2. Preparation of the ethanolic extract of yellow hawthorn fruit

Yellow hawthorn fruits were collected from Akarca Village of Nevşehir, Türkiye, in August 2023, soaked in pure water for 30 hours and dried in an oven at 55°C. After three days of drying in oven, they were cut into small pieces with a knife and their seeds were removed. 20 grams of the cut yellow hawthorn fruits were weighed for extraction and were placed on filter paper. The filter paper was closed with staples from both ends and placed in the Soxhlet extractor apparatus. 200 mL of absolute ethanol was added to the filter paper in a ratio of 1/10 to the amount of yellow hawthorn. The heating mantle was set to 80°C and the temperature was measured with a thermometer at certain intervals. The extract of yellow hawthorn fruits was taken in the Soxhlet extractor for a total of 2 cycles approximately for 6 hours. Then, excess solvent was removed under low pressure at 140 rpm at 35°C by suing rotary evaporator (19). In order to use further in vitro tests, the extract was lyophilized for 2 days at 0.05 mBar and -52°C.

2.3. Cell Culture and Maintenance

For cell culture experiments, K562 cells and MOLM-13 cells were grown in RPMI medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin and incubated in a 5% CO_2 incubator at 37°C. When 80% cell density (approximately 2-3 days) was observed in the Petri, the medium of the cells was changed and passaged.

2.4. Cell Viability Assay

The antiproliferative effect of yellow hawthorn extract on K562 and MOLM-13 cells was investigated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) cell viability assay. The MTT assay is carried out with different concentrations (100 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL, 2000 µg/mL, 3000 µg/mL, 4000 µg/mL, 5000 µg/mL, 10000 µg/mL). The cell viability assay for each cell line and for each incubation time was performed in triplicate. The cells were seeded in 96-well plates at $1x10^4$ density per well in 100 µL. The determined concentration of yellow hawthorn extract was added to each well and incubated for 24, 48 and 72 hours. Following the incubation, 10 µL of MTT dye was added to each well and incubated for 2 hours at 37°C and 5% CO_2. After incubation, 100 µL of dimethylsulfoxide (DMSO) was added to each well to dissolve the formazan crystals and then incubated for 15 minutes on a waving shaker in the dark. Later, the absorbance was determined at 570 nm by using a Varioskan LUX microplate reader (Thermo Scientific) to measure the amount of formazan crystals, and a graph of the absorbance values obtained was created (28).

2.5. Cell Death Assay

The concentrations for cell death assays were selected as 9000

µg/mL and 15000 µg/mL for K562 cells, and 3000 µg/mL and 10000 µg/mL for MOLM-13 cells. The cells were seeded in 6-well plates as $5x10^5$ cells per well in 2 mL and incubated for 48 hours. After incubation, the cells were centrifuged at 300 × g for 5 minutes at 4°C and the supernatant was discarded after centrifugation. The resulting pellet was washed with 1 mL of PBS and then centrifuged at 300 x g for 5 minutes at 4°C. This step was repeated twice. After centrifugation, the supernatant was discarded, and the resulting pellet was solubilized with 200 µL of control and 100 µL of Annexin binding solution. After the pellet was homogenized, 2.5 µL Annexin V and 2.5 µL PI were added and incubated at room temperature in the dark for 15 minutes. After incubation, 400 µL Annexin binding solution was added to each tube. Finally, the cells were analyzed by BD LSRFortessa (Becton Dickinson) flow cytometry (28).

2.6. Cell Cycle Analysis

The same concentrations were used for the cell cycle experiment as in the apoptosis assay. The cells were seeded in 6-well plates as $1x10^6$ in 2 mL per well and incubated for 48 hours. The cells were centrifuged at 260 × g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was homogenized by adding 1 mL of cold PBS and centrifuged again at 260 x g for 10 minutes at 4°C and this process was repeated twice. After centrifugation, 1 mL of cold PBS and 4 mL of cold ethanol were added to the cells and the cells were incubated at -20°C overnight for cell fixation. The next day, the cell suspension was centrifuged at 260 × g for 10 minutes at 4°C and the supernatant was discarded. The resulting pellet was dissolved with 1 mL of cold PBS and centrifuged again at 260 × g for 10 minutes at 4°C. Lastly, the supernatant was removed, and the pellet was homogenized by adding 1 mL of 0.1% Triton-X and 100 µL of RNAase (200 µg/mL) and incubated at 37°C for 30 minutes. After incubation, 25 µL of PI was added to the samples and incubated at room temperature for 15 minutes. Then, the samples were analyzed by BD Biosciences LSRFORTESS Cell Analyzer flow cytometry (29).

2.7. Statistical Analysis

The results are shown as mean±standard deviation. Statistical analysis was prepared using the GraphPad Prism 8.0.2 program. The statistical significance of the results was calculated using one-way analysis of variance (ANOVA). The value of $P \leq 0.05$ was considered statistically significant and the value of $P \leq 0.001$ was considered highly statistically significant.

3. Results

3.1. Yield of Ethanolic Yellow Hawthorn Fruit Extract

The yield is calculated by using general formula (30, 31).

$$yield = \frac{weight\ of\ dry\ extract}{weight\ of\ dry\ plant}\ X\ 100\%$$

The dried yellow hawthorn fruit weighed as 20 grams. After the extraction and powder production process in a freeze-dryer, the powder from of extract is weighed as 4 grams. According to these amounts, the yield was calculated as 20%.

$$yield = \frac{4\ grams\ of\ yellow\ hawthorn\ fruit\ extract}{20\ grams\ of\ dried\ yellow\ hawthorn\ fruit}\ X\ 100\% = 20\%$$

3.2. Ethanolic Yellow Hawthorn Fruit Extract Significantly Inhibited the Cell Proliferation of K562 and MOLM-13 cells

First, the effect of yellow hawthorn ethanolic extract on the proliferation of K562 and MOLM-13 cells was studied. Cells were treated with increasing doses of yellow hawthorn ethanolic extract for 24, 48 and 72 hours. As shown, yellow hawthorn ethanolic extract reduced the proliferation of K562 cells and MOLM-13 cells in a dose- and time-dependent manner (Fig. 1 and Fig. 2). From these dose-time curves, the IC50 values were calculated separately for 3 different treatment times. The IC50 value of K562 cells treated with yellow hawthorn extract for 24, 48 and 72 hours was calculated as 15948 µg/mL (Fig. 1, left). 9144 µg/mL (Fig. 1, middle) and 4858 µg/mL (Fig. 1, right), respectively. Among these results, IC50 value of 24 hours treatment was calculated as an approximate value, since cell viability was not observed under 50%. According to the results, the viability of K562 cells treated with yellow hawthorn extract decreased as the dose of yellow hawthorn extract increased. The IC50 value of K562 cells treated with 3 different periods decreased as well, with prolonged treatment. Thus, it was concluded that yellow hawthorn extract has a significant antiproliferative effect on K562 cells. On the other hand, MOLM-13 cells' viability was also significantly reduced in a dose and time-dependent manner. According to dose-time curves for MOLM-13 cells, IC50 values of treating MOLM-13 cells with yellow hawthorn ethanolic extract in 3 different time manners, which are 24, 48, and 72 hours, are 11772 µg/mL, 3515 µg/mL, and 1240 µg/mL, respectively (Fig. 2, left, middle, right, respectively). Similar to that of the K562 cell lines, IC50 value of 24 hours treatment was calculated as an approximate value, since cell viability was not observed under 50%. These results showed that yellow hawthorn ethanolic extract has an antiproliferative effect on MOLM-13 cells in a time- and dose-dependent manner. Our results demonstrate that ethanolic extract of yellow hawthorn shows better inhibitory effect with lower IC50 values on MOLM-13 cells compared to K562 cells for every incubation time periods.

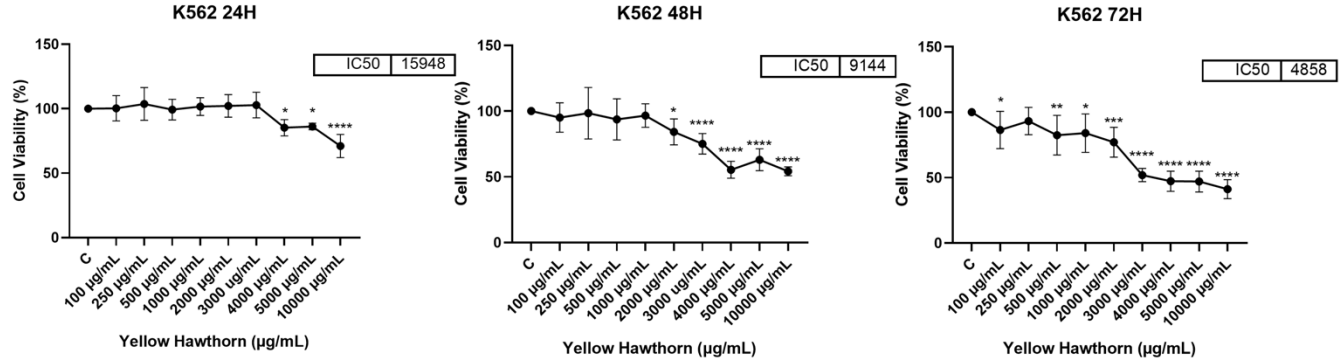

Fig. 1. Antiproliferative effect of ethanolic extract of yellow hawthorn fruit in K562 cell line at increasing doses for 24, 48, and 72 hours. Standard deviation was calculated according to the number of replicates and 3 independent replicates were made. All data are presented as mean ± S.D. (ns = P > 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

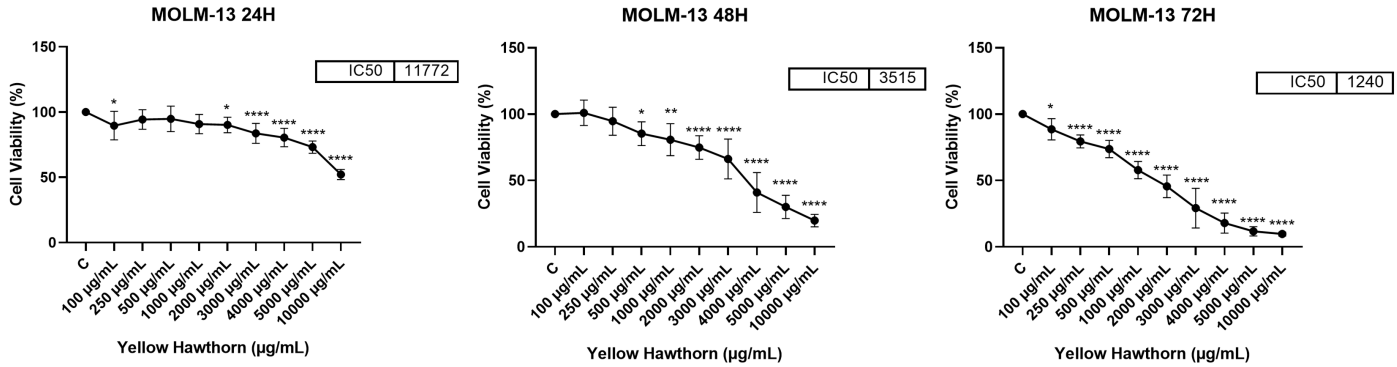

Fig. 2. Antiproliferative effect of ethanolic extract of yellow hawthorn fruit in MOLM-13 cell line at increasing doses for 24, 48, and 72 hours. Standard deviation was calculated according to the number of replicates and 3 independent replicates were made. All data are presented as mean ± S.D. (ns = P > 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

3.3. Yellow Hawthorn Ethanolic Extract Induced Apoptotic Cell death in CML and AML cells

K562 cells were treated with yellow hawthorn ethanolic extract for 48 hours at 9000 µg/mL and 15000 µg/mL, and MOLM-13 cells were treated with 3000 µg/mL and 10000 µg/mL. According to the results, it was shown that yellow hawthorn extract caused apoptotic cell death in K562 cells and MOLM-13 cells with increasing doses of yellow hawthorn extract. K562 cells treated with 15000 µg/mL yellow hawthorn extract increased the ethanolic apoptotic cell amount from 7.45% to 56.15% compared to the control (Fig.3, a). It is observed that ethanolic yellow hawthorn extract increased apoptosis by 4.4 times at the dose of 9000 µg/mL and by 12.7 times at the dose of 15000 µg/mL compared to the control (Fig. 3, b). On the other hand, late and early apoptotic cell population was determined and it was shown that yellow hawthorn extract caused a significant increase in late apoptotic cell population of K562 cells. According to the results, yellow hawthorn extract increased the amount of K562 cells undergoing late apoptosis from 2% to 45.2% at the dose of 15000 µg/mL compared to the control (Fig. 3, a). Furthermore, the viability of K562 cells decreased from 91.15% to 39.2% compared to the control. And also, as shown in the graph, the number of cells undergoing late apoptosis increased by 22.7 times compared to the control (Fig. 3, c). According to the apoptosis test results, it was determined that the ethanolic extract of yellow hawthorn fruit caused apoptosis in K562 cells. It was determined that increasing doses of yellow hawthorn extract significantly led to late apoptosis of K562 cells. On the other hand, it is indicated that at 10000 µg/mL, MOLM-13 cells undergo apoptotic cell death. It is indicated that with 10000 µg/mL dose of yellow hawthorn, ethanolic apoptotic (Q2+Q4) cell death is increased from 8.9% to 54.7% compared to the control. Moreover, the viability of MOLM-13 cells has decreased from 90.8% to 41.35% (Fig. 4, a). Furthermore, the total apoptotic cell death of MOLM-13 cells is increased by 8.87 times compared to the control (Fig. 4, b). It is shown that late apoptotic cell death is increased by 11.5 times with a dose of 10000 µg/mL as compared to the control. In addition to that, there is an increase in early apoptotic cell death, which is 7.8 times compared to the control group (Fig. 4, c). These results demonstrate that yellow hawthorn ethanolic extract causes apoptotic cell death in MOLM-13 cells and as the dose increases, the apoptotic cell death increases. Our data indicated that K562 cells are more sensitive and demonstrate a higher level of apoptosis compared to MOLM-13 cells.

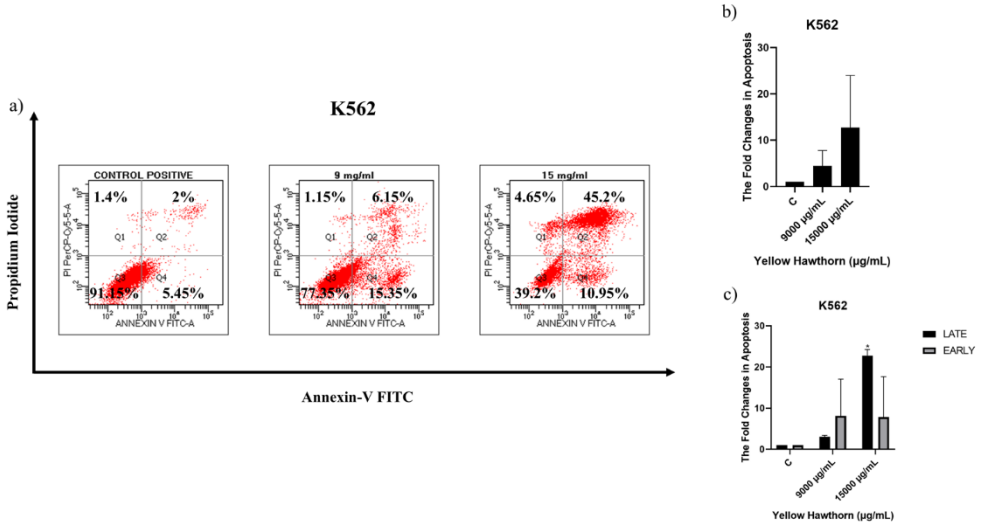

Fig. 3. a) Effect of yellow hawthorn ethanolic extract on apoptotic cell death **b)** Sample histogram of the effect of graphs on total apoptosis **c)** Sample histogram of the effect of graphs on late and early apoptosis. K562 cells were treated with yellow hawthorn ethanolic extract for 48 hours. Apoptosis analysis results and cell percentages are shown. Two independent repetitions were performed, and the results were combined and analyzed. The upper right part (Q2) of the graphs shown in Fig3 a) shows the number of cells that underwent late apoptosis, and the lower right part (Q4) shows the number of cells that underwent early apoptosis. The histogram shown in b) shows the number of cells that underwent total apoptosis (Q2+Q4). All data are presented as mean ± S.D. (ns = P > 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

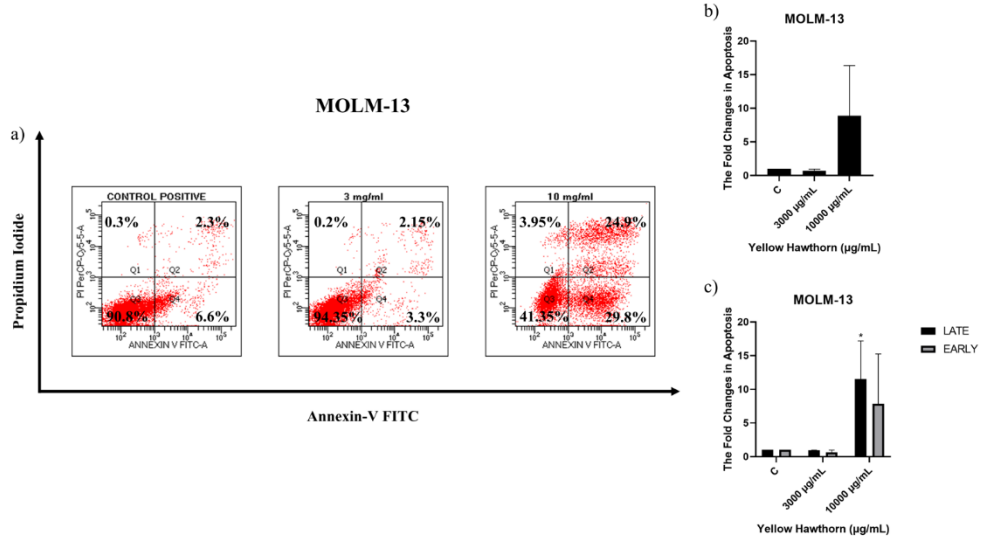

Fig. 4. a) Effect of yellow hawthorn ethanolic extract on apoptotic cell death b) Sample histogram of the effect of graphs on total apoptosis c) Sample histogram of the effect of graphs on late and early apoptosis. MOLM-13 cells were treated with yellow hawthorn ethanolic extract for 48 hours. Apoptosis analysis results and cell percentages are shown. Two independent repetitions were performed, and the results were combined and analyzed. The upper right part (Q2) of the graphs shown in Fig4 a) shows the number of cells that underwent late apoptosis, and the lower right part (Q4) shows the number of cells that underwent early apoptosis. The histogram shown in b) shows the number of cells that underwent total apoptosis (Q2+Q4). All data are presented as mean ± S.D. (ns = P > 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

3.4. Effect of Yellow Hawthorn Ethanolic Extract on Cell Cycle

Cell cycle experiments of K562 and MOLM-13 cells treated with yellow hawthorn extract were conducted with the same doses as indicated in the apoptosis assay. According to the given graph, the 9000 µg/mL concentration significantly arrested K562 cells treated with yellow hawthorn ethanolic extract in the G2/M phase and increased the cell percentage in this phase from 18.8% to 80.3% (Fig. 5, a and b). On the other hand, according to the histogram, yellow hawthorn extract caused an increase in the number of cells in the S phase of K562 cells from 15.35% to 30.8% with 15000 µg/mL (Fig. 5, a). Furthermore, the dose of 15000 µg/mL increased the number of cells in the G0/G1 phase from 4.35% to 38.2% compared to 9000 µg/mL (Fig. 5, a). On the other hand, MOLM-13 cells are arrested at the GO/G1 cell cycle phase with both 3000 µg/mL and 10000 µg/mL doses of yellow hawthorn ethanolic extract. Cell population in the G0/G1 phase increased by 53.1% to 70.6% for 3000 µg/mL, and 71% for 10000 µg/mL compared to the control (Fig. 6, a). The histogram shows that yellow hawthorn ethanolic extract has arrested the G0/G1 phase of the MOLM-13 cell line significantly, both with 3000 µg/mL and 10000 µg/mL (Fig. 6, b). Thus, it is indicated that yellow hawthorn ethanolic extract effects of cell cycle arrest on both K562 and MOLM-13 cells in different phases of the cell cycle.

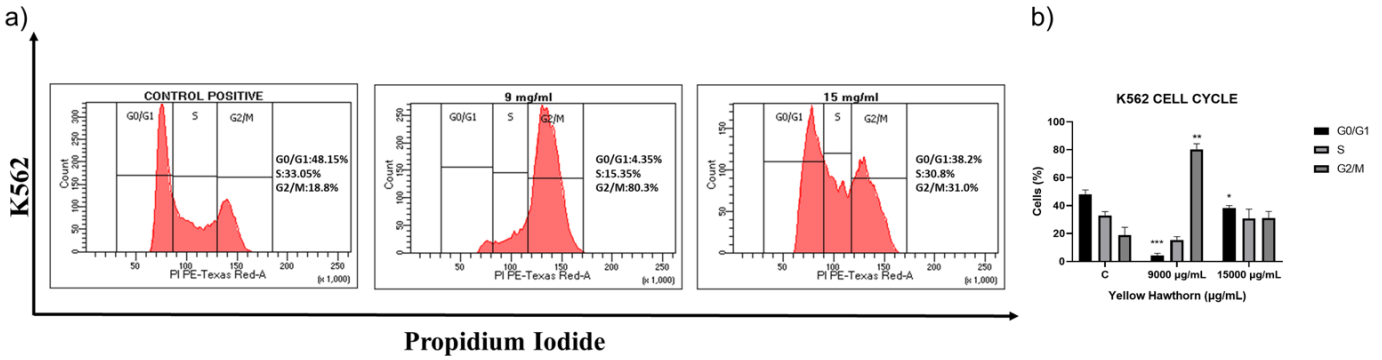

Fig. 5. a) Effect of yellow hawthorn ethanolic extract on cell cycle b) sample histogram of graphs. K562 cells were treated with yellow hawthorn ethanolic extract for 48 hours. Cell cycle analysis results are shown in Fig.5. Two independent replicates were performed and the results were combined and analyzed. All data are presented as mean ± S.D. (ns = P > 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

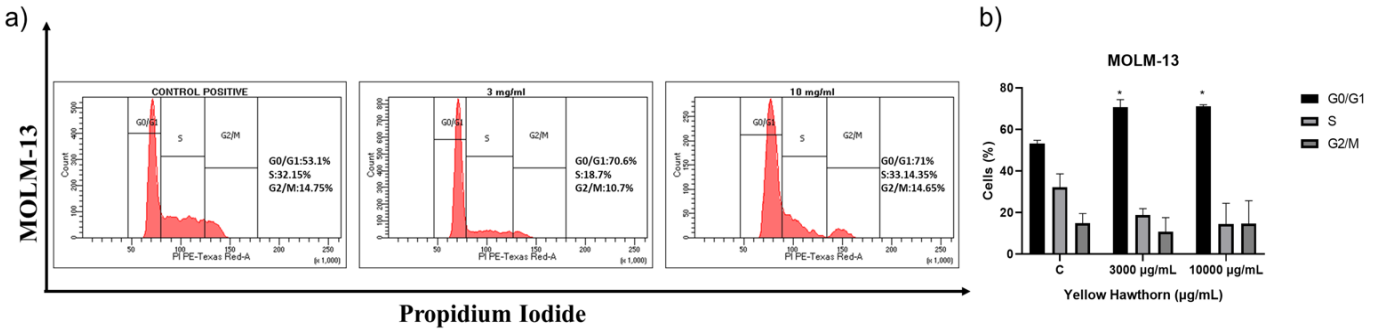

Fig. 6. a) Effect of yellow hawthorn ethanolic extract on cell cycle b) sample histogram of graphs. MOLM-13 cells were treated with yellow hawthorn ethanolic extract for 48 hours. Cell cycle analysis results are shown in Fig.5. Two independent replicates were performed and the results were combined and analyzed. All data are presented as mean ± S.D. (ns = P > 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001)

4. Discussion

Chronic myeloid leukemia (CML) is a myeloproliferative and cancer type that occurs as a result of the formation of the BCR-ABL1 oncoprotein (3). Acute myeloid leukemia (AML) is a type of hematological cancer which is characterized by the differentiation of hematopoietic stem cells into myeloid cells (8, 9).

Tyrosine kinase inhibitors are used in both CML and AML treatment. In CML, these inhibitors inhibit BCR-ABL1 and break the resistance caused by mutations caused by the BCR-ABL1 oncoprotein (32, 33). On the other hand, these inhibitors are used in AML to inhibit the FLT3 gene which is a tyrosine kinase mutation that activates downstream pathways (34, 35). However, patients with CML and AML develop resistance to tyrosine kinase inhibitors over time and regress treatment (36-38).

Traditional treatment methods have been used for centuries in countries and use natural products and their bioactive compounds (39). Natural products and isolated compounds from them are promising for drug discovery and development in cancer treatment (40). Paclitaxel is an example of an anticancer drug that is an isolated alkaloid (41). Especially, secondary metabolites found in natural products have anticancer effects as they regulate apoptosis, cell cycle, and proteins altered in pathways (42). Natural products are used in the treatment of different diseases and especially, in cancer treatment, as natural products have anti-inflammatory, antioxidant, and antitumor effects. (43). According to studies, it has been determined that compounds such as flavonoids, terpenes, alkaloids, etc. in natural products can also reverse multiple drug resistance (MDR) by regulating signaling pathways and related proteins (44). Ursolic acid which is a type of terpenes and widely found in hawthorn fruit, and it decreases the proliferation and invasion of human glioblastoma cells by downregulating the PI3K/Akt pathway (45, 46). On the other hand, flavonoids have anticancer ability by inhibiting the metabolic activation of cancer cells and downregulation of MAPK, FAK, and PI3K/Akt pathways (47). It is reported that the proliferation of MCF-7 cells which are breast cancer cells is suppressed, and apoptosis is induced by isoflavones (48).

This study examines the anticancer effect of the ethanolic extract of yellow hawthorn fruit on CML and AML cancer cell lines with respect to the induction of cell death and cell cycle arrest. In our study, ethanolic extract of yellow hawthorn significantly decreased the proliferation of MOLM-13 and K562 cell lines in a dose- and time-dependent manner, which is conducted for the first time. However, for the K562 cell line, the IC50 values for time periods are high compared to the MOLM-13 cell line's IC50 values. Similar to our results, it has been reported that the ethanolic extract of hawthorn fruits showed an anticancer effect by inhibiting the proliferation of DLD-1, a colorectal cancer cell line (19). Another study

demonstrated that the proliferation of HCT116 cells is inhibited with the treatment of polysaccharides extracted from hawthorn (26, 27).

As a further study, we continued analyzing the effect of ethanolic yellow extract on apoptosis. According to our data, K562 showed a better apoptotic response than MOLM-13 as their fold changes in apoptosis are 12.7- and 8.87-fold changes. Consistent with our data, Ma et al. reported that apoptosis is induced as apoptotic cell amounts are increased in a dose-dependent manner with the treatment of hawthorn extract in HCT116 cells with a lower dose range (125-1000 µg/mL) (26). Furthermore, the study by Zhou et al. revealed that in liver cancer cell lines, it has been investigated the proliferation of cells is suppressed and apoptotic protein levels are increased. Similarly, this study used the ethanolic extract of hawthorn fruits. Also, the anti-apoptotic protein BCL-2 level is decreased, and the apoptotic protein BAX level is increased, and apoptosis is induced by cleaving the caspase-9 and caspase-8 (21). On the other hand, it has been reported that methanolic hawthorn berry extract reduced the proliferation of glioblastoma cells with increasing PARP-1 levels, resulting in apoptosis induction (49, 50).

To further investigate, we examined the effect of the ethanolic extract on the cell cycle and our results demonstrated that the ethanolic extract induced cell cycle arrest at G2/M phase in K562 cells and the cell population is increased to 80.3% with treatment of 9000 µg/mL. In the same manner, in MOLM-13 cells, the G0/G1 phase is arrested with the treatment of ethanolic hawthorn extract and cells in this phase are increased to 70.6% (3000 µg/mL) and 71% (10000 µg/mL), respectively. The results of this study can be concluded that the ethanolic extract of yellow hawthorn fruit demonstrates the anticancer effect on CML and AML cell lines in terms of a decrease in cell viability, induction of apoptosis and cell cycle arrest. Similar to our data, it has been reported that hawthorn extract arrested cell cycle by a decrease in CDK and cyclin levels, also, p21 expression is increased and G1, S, and G2/M phases arrests are investigated (21). Similarly, Zhang et al. reported that hawthorn extract arrests the cell cycle in the S phase in colon cancer cells, and apoptosis is also increased in these cells. However, this hawthorn extract shows these biological responses with a lower dose (1000 µg/mL) compared to our doses (22). Taken together, we demonstrate that two cell lines with different genetic backgrounds could respond differently to the same yellow hawthorn extract in different biological assays. Two AML cells respond differently to the treatment because K562 and MOLM-13 cell lines belong to different types of hematological malignancies. These cells have different neoplastic transformations that drive their survival and unlimited division. Having different genetic backgrounds activates different signaling pathways, and thus different survival mechanisms would be induced. This could be a reason behind the different responses of these cell lines in terms of apoptosis and cell cycle arrest. In addition to genetic background differences, epigenetic regulation mechanisms could be different, which would have an impact on the response of cells to the same treatment. Besides, the metabolic activities of these cell lines could be variable due to their different FLT3 expression or Bcr-Abl expression profiles for MOLM-13 and K562, respectively. The different oncoproteins may lead to different escape mechanisms for these cell lines. We believe the merit of this study is to reveal the effect of the used natural product on different types of leukemia cells and although a consistent antiproliferative, antiapoptotic and cytostatic effect was observed for both cell lines, further studies should be conducted to reveal the differences between the cell lines.

In this study, the anticancer effect of yellow hawthorn fruit was investigated on chronic myeloid leukemia K562 and acute myeloid leukemia MOLM-13 cells. This study is novel as anticancer effect of yellow hawthorn fruit ethanolic extract has not been investigated before on K562 and MOLM-13 cell lines. Also, it is valuable to examine the effect of hawthorn fruit as an ethanolic extract in cancer treatment as it consists of many bioactive compounds and ethanolic extract gives efficient results compared to single isolated phytochemicals in cancer treatment.

In conclusion, ethanolic extract of yellow hawthorn fruit showed antiproliferative, apoptotic and cytostatic effects on MOLM-13 and K562 cell lines in a dose- and time-dependent manner. Furthermore, the ethanolic yellow hawthorn fruit extract induced apoptosis significantly and arrested the cell cycle in the G2/M phase in K562 and G0/G1 phase in MOLM-13 cells. As a future perspective, a mechanistic follow-up study should be conducted to reveal the underlying mechanism for apoptotic cell death. Some apoptotic and antiapoptotic marker proteins should be detected in response to the treatment of cell lines. In addition, the cell cycle regulatory mechanism should be detected by checking the expression levels of phase specific cyclin dependent kinases or cyclins. The G2/M cell cycle arrests may be due to microtubule disorganization and thus, a follow-up for our study would be further investigations on cytoskeletal organization upon extract treatment. Moreover, even though the motivation of our study was to use a natural product to have anticancer effect on cancer cells, aiming a minimal side effect for healthy cells, this should also be demonstrated using healthy controls for leukemia cells. Finally, the in vivo studies should be designed to show the anticancer effect in leukemia models. Lastly, although the results of the current study require further validation, we believe it will be a notable study for the demonstration of antiproliferative, apoptotic effects and cytostatic effects of the specific natural product on AML and CML cell lines. Further experiments are required for a better understanding of the effect and mechanism of yellow hawthorn ethanolic extract on these cell lines.

Ethical Statement

The authors declare that no ethics committee approval is required for this study.

Conflict of interest

The authors declare no conflict of interest.

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Authors' contributions

Concept: İ.A., Design: İ.A., Data Collection and Processing: A.N.A, Analysis or Interpretation: İ.A., A.N.A, Literature Research: İ.A., A.N.A, Writing: İ.A., A.N.A.

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