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Ficus carica extract causes cell cycle arrest and induces apoptosis in MG-63 and HT-29 cancer cell lines

Ficus carica ekstraktı, hücre döngüsünün durmasına neden olur ve MG-63 ve HT-29 kanser hücre hatlarında apoptozu indükler

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ABSTRACT

Objective: *Ficus carica* (Fig) is a leafy tree of Moraceae, which is used in local traditional medicine to treat various diseases. Recent studies revealed an impressive anti-cancer efficiency of *F. carica* extracts in different types of cancer cells. This study aimed to investigate the anti-cancer effects of *F. carica* extract on colon cancer cell line HT-29 and bone cancer cell line MG-63.

Methods: The human colon cancer cell line HT-29 and bone cancer cell line MG-63 were used to investigate the effects of *F. carica* extract. The effects of *F. carica* on cell viability were evaluated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] assay. Complementary analyses for evaluating membrane integrity and toxicity were performed by estimating LDH (lactate dehydrogenase) assay. Mechanisms of cell death were analyzed using MuseTM Annexin-V and TUNEL assays. Cell-cycle distribution was examined using flow cytometri analysis.

Results: The results demonstrated that *F. carica* extract caused a significant decrease in cell viability in cancer cells in a dose-and time-dependent fashion, but

ÖZET

Amaç: *Ficus carica* (Fig), geleneksel tıpta çeşitli hastalıkları tedavi etmek için kullanılan yapraklı bir Moraceae ağacıdır. Son araştırmalar, farklı kanser hücrelerinde *F. carica* özlerinin etkileyici bir anti-kanser etkinliğini ortaya çıkardı. Bu çalışma, *F. carica* ekstraktının kolon kanseri hücre hattı HT-29 ve kemik kanseri hücre hattı MG-63 üzerindeki anti-kanser etkilerini araştırmayı amaçladı.

Yöntem: *F. carica* ekstraktının etkilerini araştırmak için insan kolon kanseri hücre hattı HT-29 ve kemik kanseri hücre hattı MG-63 kullanıldı. *F. carica*'nın hücre canlılığı üzerindeki etkileri, MTT [3-(4,5-dimetilthiyazol-2-il)-2,5 difeniltetrazolyum bromür] metodu kullanılarak değerlendirildi. LDH (laktat dehidrogenaz) metodu ile membran bütünlüğünü ve toksisiteyi değerlendirmek için tamamlayıcı analizler yapıldı. Hücre ölümü mekanizmaları, MuseTM Annexin-V ve TUNEL testleri kullanılarak analiz edildi. Hücre döngüsü dağılımı, akış sitometri analizi kullanılarak incelendi.

Bulgular: Elde edilen sonuçlar, *F. carica* ekstraktının kanser hücrelerinde doza ve zamana bağlı bir şekilde hücre canlılığında önemli bir azalmaya neden olduğunu

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not in the healthy cell line. According to MTT analysis, the optimal activity of *F. carica* in MG-63 and HT-29 cell lines was determined in 48 h at 1:100 dilution and 24 h at 1:10 dilution, respectively. In addition, the cell viability did not drop below 50% in the hFOB 1.19 cell line at any dose and time interval, indicating that *F. carica* was not cytotoxic to normal cells. The LDH activity in culture media showed that *F. carica* had cytotoxic effects on cancer cell lines. A significant increase in LDH activity in the cultured media through the loss of membrane integrity during apoptosis pathways was observed in cancer cells, whereas the extract did not have a cytotoxic effect in the control cell line. According to HPLC analysis, *F. carica* used in this study contained 8.17063x10-1 mg/L protocathecuic acid. Cell cycle analysis showed that the *F. carica* extract did not affect the MG-63 cell line, but caused an arrest at the S phase in HT-29 cells. In TUNEL assay, fluorescent staining was detected in cancer cell lines that underwent apoptosis, while there was no staining in control cells. The Annexin-V and Dead Cell Assay confirmed apoptosis in both cancer cell lines.

Conclusion: *F. carica* was found to have anti-cancer effects on both colon and bone cancer cell lines. While *F. carica* extract can activate different types of cell death in cancer cell lines, it did not cause any cytotoxic effects on healthy cells. A possible mechanism for the anti-cancer activity of *F. carica* is through induction of apoptosis as observed in the colon cancer cell line HT-29 and bone cancer cell line MG-63.

Key Words: Anti-cancer, apoptosis, cell death, HT-29, colon cancer, *F. carica* extract, MG-63, osteosarcoma

göstermektedir. MTT analizine göre *F. carica*'nın MG-63 ve HT-29 hücre hatlarında optimal aktivitesi sırasıyla 48 saatte 1:100 dilüsyonda ve 24 saatte 1:10 dilüsyonda belirlendi. Ayrıca hücre canlılığı, hFOB 1.19 hücre hattında herhangi bir doz ve zaman aralığında %50'nin altına düşmedi, bu da *F. carica*'nın normal hücreler için sitotoksik olmadığını gösterir. LDH aktivitesi, *F. carica*'nın kanser hücre hatları üzerinde sitotoksik etkileri olduğunu gösterdi. Apoptoz sırasında kanser hücrelerinde membran bütünlüğünün kaybı yoluyla LDH aktivitesinde önemli bir artış gözlenirken, ekstrakt kontrol hücre hattında sitotoksik bir etkiye sahip değildi. HPLC analizine göre bu çalışmada kullanılan *F. carica*'nın, 8.17063x10-1 mg/L protokateşuik asit içerdiği tespit edildi. Hücre döngüsü analizi, *F. carica* ekstraktının MG-63 hücre hattını etkilemediğini, ancak HT-29 hücrelerinde S fazında bir durmaya neden olduğunu gösterdi. TUNEL testinde, kontrol hücrelerinde floresan boyanma olmazken, apoptoz geçiren kanser hücre hatlarında floresan boyama tespit edildi. Annexin-V ve Dead Cell Assay, her iki kanser hücre hattında da apoptozu doğruladı.

Sonuç: *F. carica*'nın hem kolon hem de kemik kanseri hücre hatları üzerinde anti-kanser etkileri olduğu bulundu. *F. carica* ekstraktı, kanser hücre dizilerinde farklı hücre ölümlerini aktive edebilirken, sağlıklı hücreler üzerinde herhangi bir sitotoksik etkiye neden olmadı. *F. carica*'nın anti-kanser aktivitesi için olası bir mekanizma, kolon kanseri hücre hattı HT-29 ve kemik kanseri hücre hattı MG-63'te gözlemlendiği gibi apoptozun indüklenmesidir.

Anahtar Kelimeler: Anti-kanser, apoptoz, hücre ölümü, HT-29, kolon kanseri, *F. carica* ekstraktı, MG-63, osteosarkom

INTRODUCTION

F. carica (Fig) is a leafy tree of Moraceae and is considered as one of the oldest fruits alongside with apples and grapes (1). *F. carica* products are widely used as food and medicine in the Middle East (2). Fruit, root and leaves are used in traditional medicine to treat various conditions such as gastrointestinal, respiratory and cardiovascular disorders (3). Although the anti-cancer effect has been shown in various

cancer cell lines as in breast, colon and liver cancers, no study on osteosarcoma has been identified yet.

According to 2018 data of the American Cancer Research Institute, colon cancer is the third most common cancer in men and the second most common cancer in women. It is also estimated that over 1.8 million new cases of colon cancer and 881,000 deaths occurred in 2018 (4). Similarly, osteosarcoma (OS) represents the most common bone cancer (incidence: 0.3 per 100 000 per year) and the incidence is higher in adolescents (4). Risk factors for OS include previous radiation treatments causing germline abnormalities such as Paget's disease, Li-Fraumeni, Werner syndrome, Rothmund-Thomson syndrome, Bloom syndrome, and hereditary retinoblastoma (5). The two most common inherited syndromes linked with colorectal cancers are familial adenomatous polyposis (FAP) and hereditary non-polyposis colo-rectal cancer (HNPCC). Lynch, Turcot and Peutz-Jeghers Syndroms may also induce colorectal cancer (6).

In this study we focused upon the effect of *F. carica* extract to observe its efficacy as anti-cancer agent on bone and colon cancer cell lines. To elaborate our study further, we analyzed the cell survival, cytotoxicity, apoptosis, cell cycle progression of these two lines after challenging them with the extracts subjected to this study.

Resistance to chemotherapy drugs and poor clinical outcome is a common encountered problem in approxi-mately 50% of cancer patients (7,8). In addition, high-dose chemotherapy causes various side effects and tumor recurrence (8). Therefore, it is necessary to develop prognostic markers and therapeutic targets in osteosarcoma in order to improve treatment approaches (9).

Medicinal plants have been the subject of therapeutic purposes past to present. According to the data of the WHO, 80% of the human population have been estimated to prefer treatments based on herbal medicines rather than synthetic medicines (10). Current treatment options based on synthetic drugs/ chemotherapy offer limited therapeutic success in cancer, as they have toxic effects, are expensive and change the functioning of the cell signaling pathways (10,11). Natural compounds, such as phytochemicals, minerals, and vitamins have emerged as promising candidates to reduce cancer burden (11).

In this study, *F. carica* extract was investigated for anti-cancer effects in terms of cytotoxicity, membrane and DNA fragmentation change, cell cycle, cell death pathways in two cancer cell lines: osteosarcoma cancer cell line (MG-63), colon cancer cell line (HT -29).

Limited information has been obtained in the literature regarding the effect of *F. carica* on apoptotic induction on cancer cell lines. In our study, it is aimed to obtain a prediction about the effect of *F. carica* apoptotic induction and cell cycle by detecting biochemical and morphological changes in order to determine the apoptotic processes of the cancer cell line.

MATERIAL and METHOD

Preparation of plant extract

F. carica extract (cas no: 90028-74-3) was commercially available from Nante Kimya (Istanbul, Turkey). The starting amount for the fractionation process is 50 ml. To prepare the aqueous fraction, it was extracted using solvents of varying polarity such as hexane, dichloromethane, methanol, ethanol, water, respectively. The extract was subjected to evaporation under nitrogen at 70°C (FMS/SuperVap 6 Concentrators). Solvents were added at a ratio of 1:20 and sonicated in an ultrasonic bath (Bandelin Electronic/Sonorex Digital 10 P). The separation funnel and the phase formed in the upper part were separated. The remainder was subjected to evaporation under 70°C nitrogen. Finally, the ultra-pure water was added to the dried extract to obtain *F. carica* aqueous fraction (12). After fractionation, the aqueous fraction of 3330 mg/ml *F. carica* extract was obtained as the main stock.

HPLC-DAD Analysis

Protocatechuic acid (Sigma Aldrich, Germany) in *F. carica* extract was determined by HPLC method by Tezcan et al. (12). Aqueous fractions were subjected to HPLC analysis using Agilent Technologies/1200 Infinity. Reverse phase analytical HPLC experiments were performed on a Thermo Fisher Scientific 5 µM C18 column (250 × 4.6mm). A variable wavelength ultraviolet visible detector was set at 235 nm. The mobile phase consisted of 1% formic acid in water (solvent A) and acetonitrile (solvent B). The standard acid was prepared as 5 ppm, 10 ppm, 20 ppm, 40 ppm, 50 ppm, 100 ppm. The flow rate was maintained column, at a rate of 5 mL/min at 25°C.

Cell Culture

Osteosarcoma cancer cell line (MG-63), colon cancer cell line (HT-29) and human bone cell line (hFOB 1.19, control cell line) were purchased from The American Type Culture Collection (ATCC, US). MG-63 cells, HT-29 cells and hFOB 1.19 cells were cultured in MEM (Sigma Aldrich, Germany), Mc-Coy's 5A (Sigma Aldrich, Germany) and DMEM/F12 (Sigma Aldrich, Germany), respectively. Media were supplemented with 10% fetal bovine serum (FBS) and 1% penicilin-streptomicin and cells were cultured at 37°C in a humified atmosphere with 5% CO_2.

Cell Viability and Cytotoxicity Analyses

MTT Assay

The cells were seeded at 5.000 cells/well in a 96 well plates and incubated in media with various concentrations of *F. carica* extract for 24, 48, or 72 h at 37°C in a humified atmosphere with 5% CO_2. The effect of *F. carica* on cell viability was assessed by MTT assay (Biotium, USA). The plates were read at 490 nm using a microplate reader (Neales/MB-530). All experiments were repeated three times at each timepoint. IC50 values of *F. carica* (IC50; inhibition value of 50% cellular proliferation) for each cell line were determined.

$$Inhibition = \frac{absofcontrol - absofsample}{absofcontrol} x100$$

Cytotoxicity Assay

The cells were seeded in a 96 well plate at a density of 25,000 cells/mL and incubated at 37°C in a humified atmosphere with 5% CO_2. The cells were treated with five serial dilutions (1:10, 1:100, 1:1000, 1:10000, 1:100000) of *F. carica* extract. Cytotoxicity assays were performed using Cytotoxicity Assay Kit (Sigma Aldrich, Germany) and NYZ Bradford Reagent (NZYTECH, Portugal). LDH assay measuring total LDH activity was carried out at three timepoints, 24 h, 48 h and 72 h. The plates were read at 595 nm using a microplate reader (Thermo Fisher Scientific/ Varioskan Flash 3001). The enzyme activity was determined as the amount of enzyme/unit protein.

Cell Cycle Analyses, TUNEL and Apoptosis Assays

Cell-cycle Analysis

Cell cycle distribution in cells treated with different concentrations of extract was evaluated for washed with cold PBS and fixed in 70% ethanol at +4°C for 30 minutes. Following, the cells were resuspended in ice cold PBS containing %1 BSA and 50µL RNase incubated at 37°C for 20 minutes, and then stained with a propidium iodide (PI) solution 37°C for 20 minutes. Cell cycle were analyzed by flow cytometry (NovoCyte Flow Cytometer Systems 1-3 Lasers) (13).

TUNEL Assay

The cells were seeded in a chamber slides (Isolab, Germany) at a density of 4000 cells/cm^2. Apoptosis was detected by the TUNEL method using the ApopTag Plus Peroxidase In Situ Apoptosis Kit (Merck Milipore, USA) according to the manufacturer's instructions. Cells were left for 15 seconds by applying 1 mL of PBS per well and washed 2 times. 200µL of 100% methanol was added per well and incubated at -20°C for 15 minutes. Methanol was removed from the wells. 1 mL of PBS was applied and left for 15 seconds then

washed 2 times. 1 mL of PBS containing 0.2% Triton X-100 was added and incubated at room temperature for 30 minutes. Cells were washed 2 times with 1 mL of PBS. Cells were incubated with 100 µL Equation Buffer for 5 minutes at room temperature. 50µL of Tunnel Reaction Buffer + 1µL of enzyme was added. 3. well Tunnel Reaction Buffer was added, no enzyme added. It was incubated at 37 °C for 80 minutes in a humid and dark environment. The cells were washed 3 times with 0.1% Triton X-100 + 5 mg / mL BSA + PBS. After each solution was added, it was left at room temperature for 5 minutes. After washing, the excitation wavelength was examined under fluorescence microscopy at 450-500 nm and scanning wavelength at 515-565 nm (Euromex/Oxion Inverso, Holland).

Annexin-V and Dead Cell Assay

After completing the incubation period, the extent of apoptosis was investigated using Muse™ Annexin-V & Dead Cell Kit (Merck Milipore, USA) with Muse cell analyzer. The assay utilizes annexin V to detect phosphatidylserine on the external membrane of apoptotic cells. Cells at a starting density of 1×10^6 cells were seeded onto regular plates and treated of *F. carica*. The cells were harvested, and 100 µL of cell suspension was added to 100 µL of the Muse™ An-nexin V & Dead Cell reagent. After 20 minutes of incubation at room temperature in the dark, the samples were analyzed according to the manufacturer's protocol. Using designated programming on the cell analyzer, the number of live, dead, early and late apoptotic cells were determined. Total apoptosis was calculated by combining the number of cells from late and early apoptosis quadrants of the histograms.

Statistical Analyses

All experimental data are presented as mean ± SD of at least two independent experiments for in vitro studies. For statistical analysis and calculations, IBM SPSS Statistics 25.0 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp), ANOVA (ANalysis Of VAriance), Mann Whitney U test, and comparisons in the number of repetitions Wallis test and MS-Excel 2016 program were used for figures. All p values <0.05 were considered statistically significant.

RESULTS

Active Compounds was present in *Ficus carica* extract

The water fraction from *F. carica* extract was prepared using polar solvents. The presence and amount of protocatechuic acid, a phenolic compound for *Ficus carica* extract, were determined with HPLC (12). The *F. carica* extract HPLC chromatogram is shown in Figure 2. In the HPLC diagram, *F. carica* extract appeared to be 8.17063x10-1 mg/L in the water fraction.

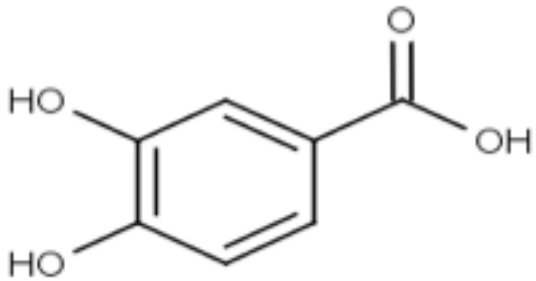


Figure 1. The chemical structure of the protocatechuic acid (3,4-dihydroxybenzoic acid)

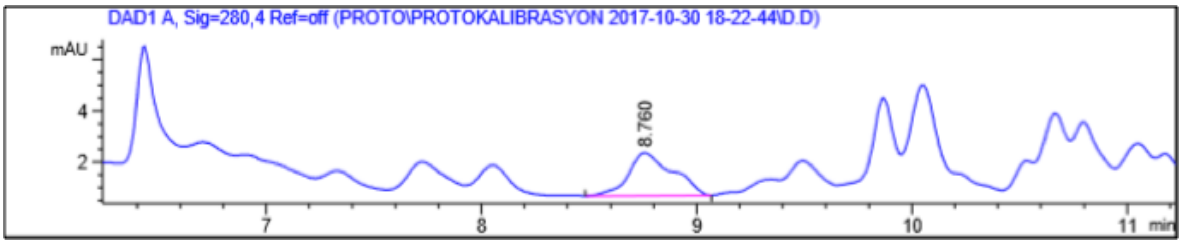
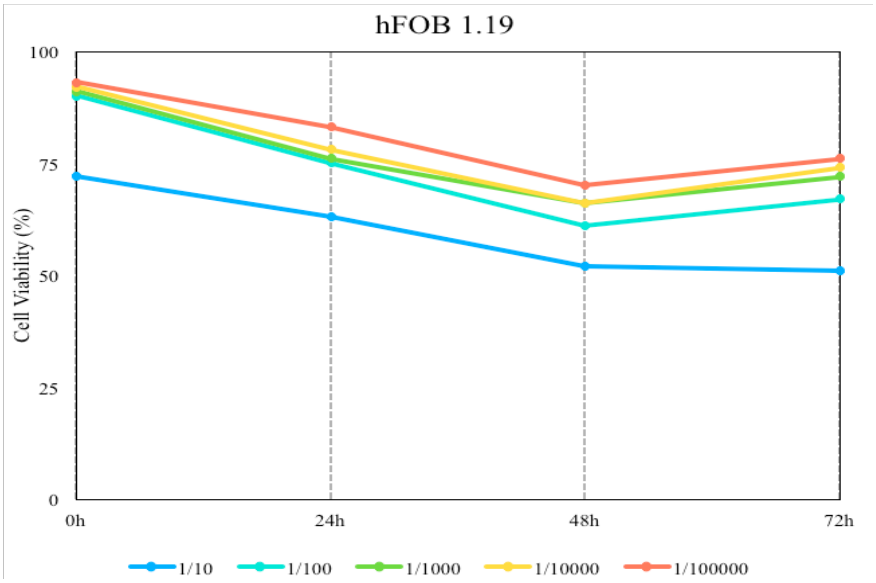


Figure 2. A representative of HPLC chromatogram of *F. carica* in extract. HPLC chromatogram of water fraction of *F. carica* in extract at 280 nm. Peak at 8,760 min. corresponding to protocatechuic acid. The first peak in the chromatographic analysis image is due to solvents in the HPLC mobile phase. However, it does not have a negative effect on the peak formed by the reference material

Treatment with extract of *F. carica* inhibited cellular proliferation of MG-63 and HT-29 cells, but not hFOB 1.19 cells

To investigate the anticancer effects of *F. carica* extracts, the inhibitory effect of MG-63, HT-29 and hFOB 1.19 was evaluated using MTT after 24-72 hour exposure of 3330 mg/ml *F. carica* at varying dilutions (prepared from the origi-nal stock). The time and dose were determined as the inhibitory concentration (IC_{50}) when 50% of the cells were dead. MTT analysis showed that *F. carica* extract treatment decreased the proliferation level in cancer cell lines MG-63 and HT-29 in a dose- and time-dependent manner (Figure 3b and 3c). IC_{50} could not be determined since the decrease in proliferation level for hFOB 1.19 cell line at any dose and time point as it was not below 50%. Although this finding suggests that *F. carica* does not have a negative effect on proliferation for the healthy cell line, future studies may need to confirm this (Figure 3a). The IC_{50} dose, time interval and proliferation percentage in MTT assay is as follow for MG-63 and HT-29 cell lines; 1:100 concentration at 48th hour, 52%, 1:10 concentration at 24th hour, 54%, respectively. The study was designed according to IC_{50} determined by MTT assay for each cell line.

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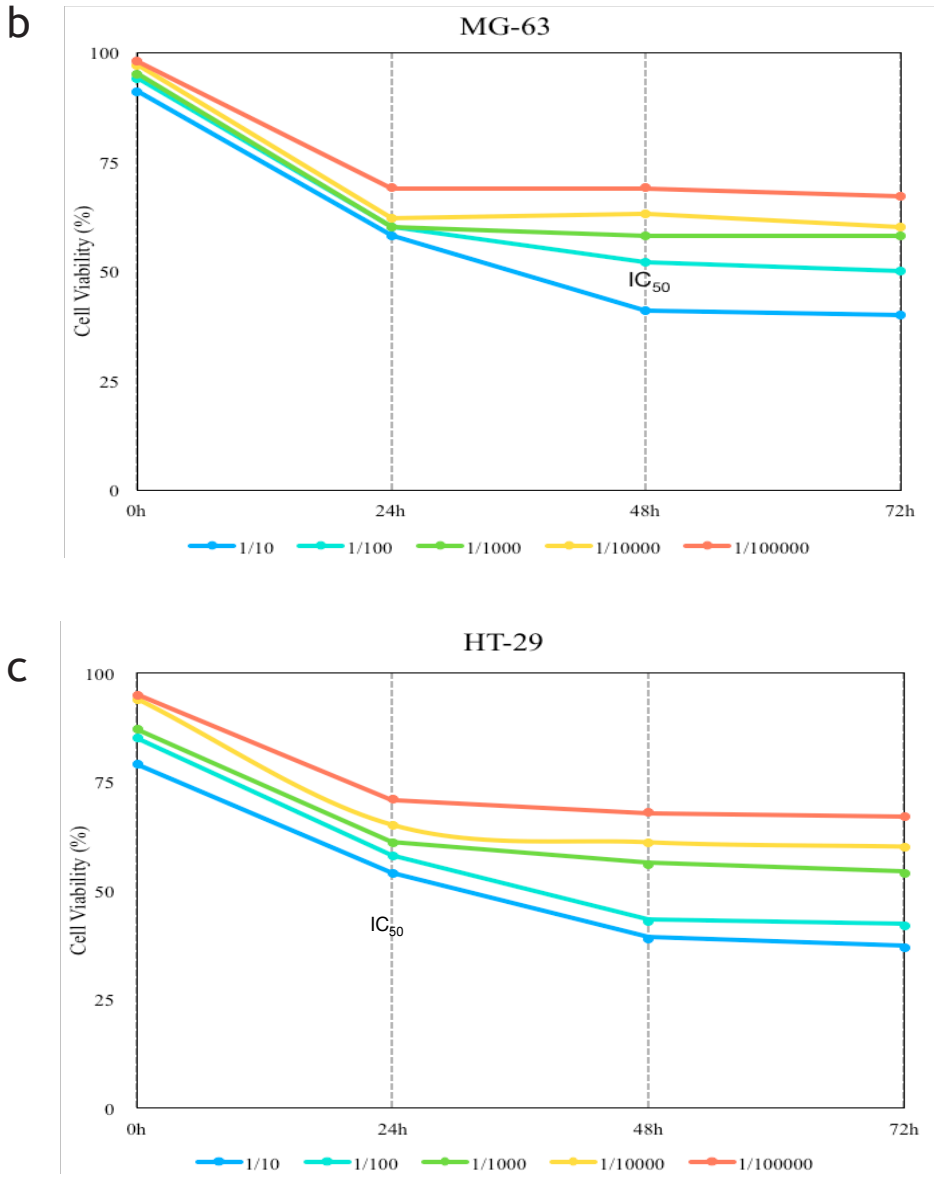
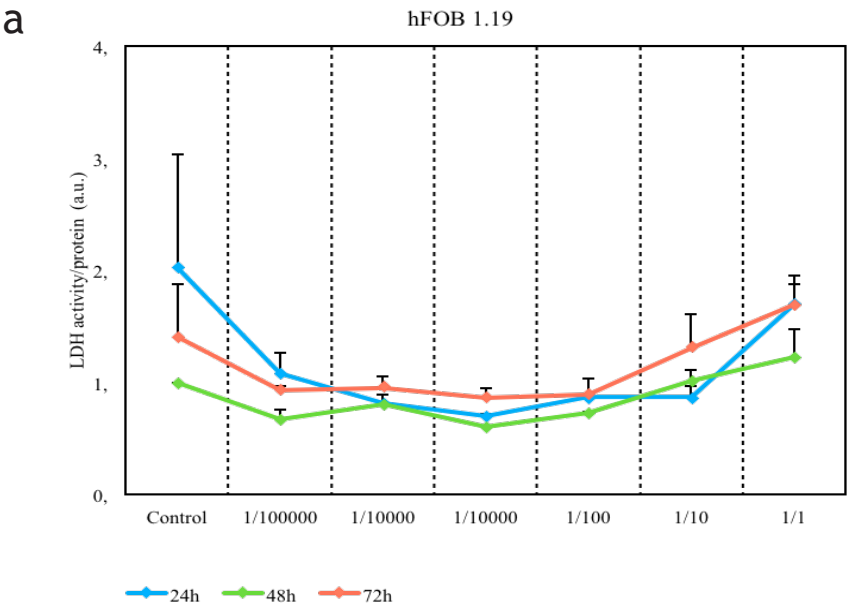


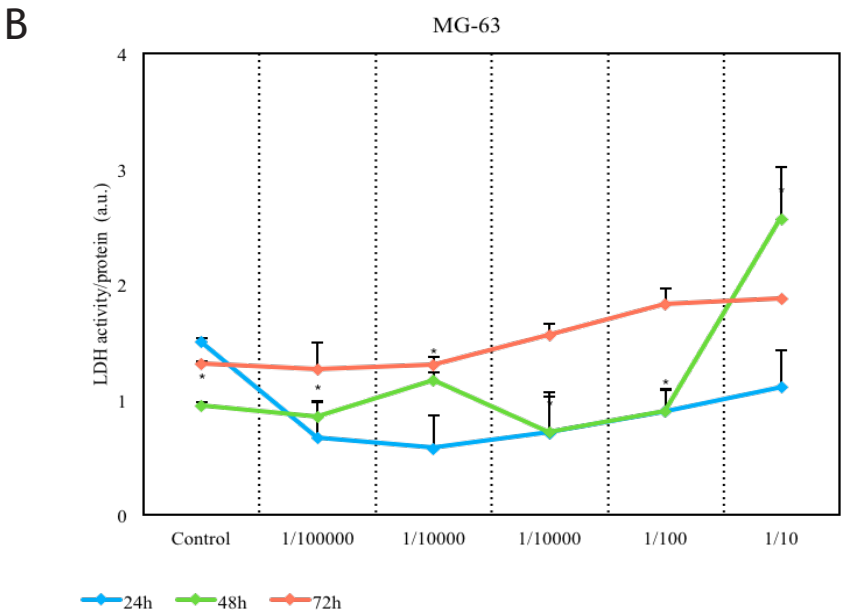
Figure 3. Cell proliferation analysis results of *Ficus carica* extract.
MTT assay was performed after treatment with *F. carica* extract for (a) hFOB 1.19, (b) MG-63 and (c) HT-29. The cells were seeded into 5000 cells/well in 96-well plates and were subjected to different concentrations over different incubation periods. All experiments were repeated in triplicates for each time-point. IC_{50} is the inhibition value of 50% cellular proliferation.

LDH assay showed the cytotoxicity of the *F. carica* extract against MG-63 and HT-29

LDH activity was determined using the LDH method and Bradford experiment as the amount of enzyme per unit protein. The extract was applied at the same time points and doses to correlate with the MTT assay results. LDH / Bradford analysis showed a decrease in LDH activity in cancer cells in a time and dose dependent manner which has a linear correlation with proliferation data. This linear decrease in the amount of LDH supported the MTT experiment results as the number of cells decreased (Figure 4a, 4b and 4c). With

increasing time and dose, LDH activity increased and toxic effect emerged, strengthening the idea that the cell's membrane structures were disrupted and the cell went into death. The results show that *F. carica* has a cytotoxic effect on cancer cell lines and there is a significant increase in LDH release into the culture medium through loss of membrane integrity during apoptosis. Thus, it was confirmed that *F. carica* was cytotoxic for cancer cells and result of LDH test was agreement with the finding of MTT result.





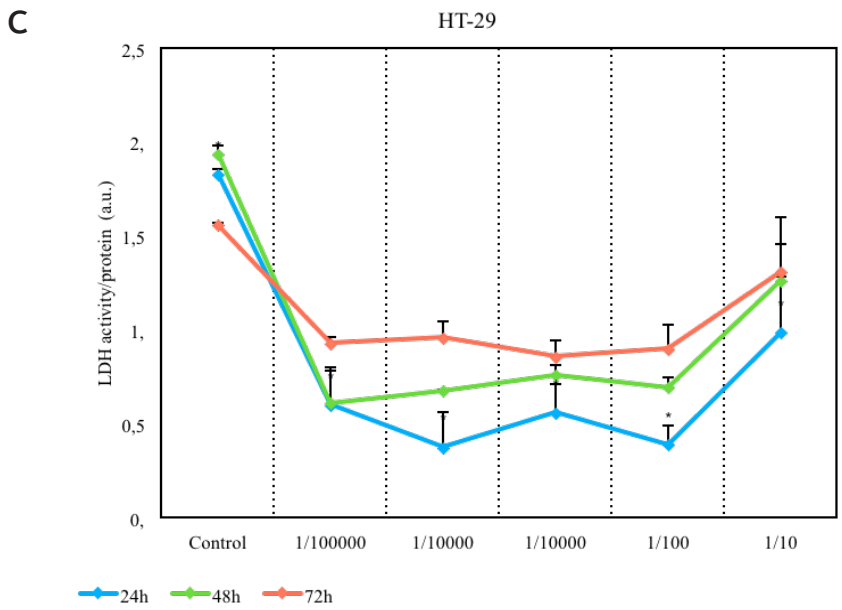
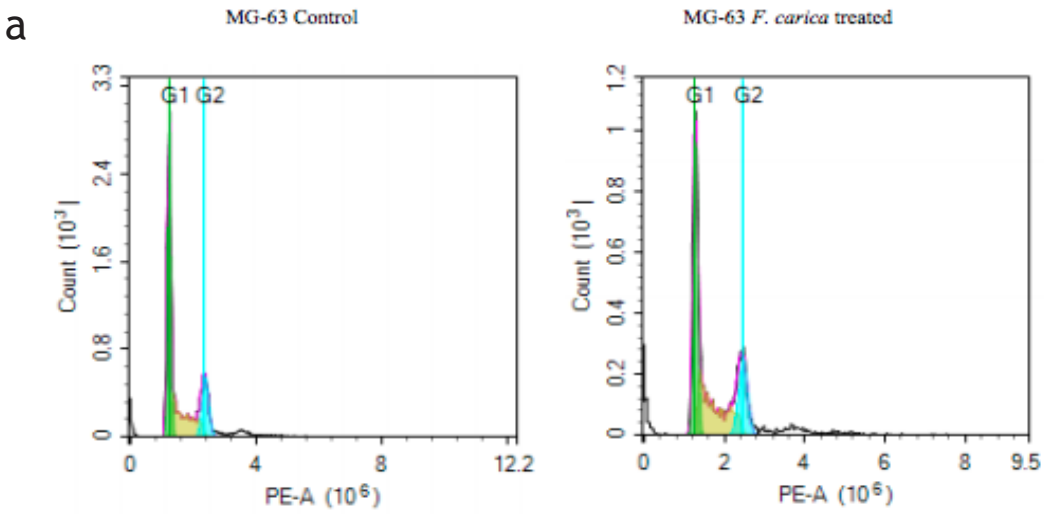


Figure 4. LDH assay showed the cytotoxicity of the *F. carica* extract against MG-63 and HT-29.
(a) hFOB 1.19, (b) MG-63 and (c) HT-29 cells were treated with extract of *F. carica* at different concentrations over different incubation period. Cytotoxicity analysis of treatment cells evaluated LDH/Bradford assays. The experiments were performed in triplicate and expressed as mean± SD values.
*Their values are different from the control groups ($p < 0.05$).

F. carica extract did not arrest the cell cycle of cells

To clarify how *F. carica* inhibits the proliferation of cancer cells, the effect of the extract on the cell cycle was examined by flow cytometry method.As shown in Figures 5a and 5b, there was no difference in percentage of cells at the G2 / M, S, and G0 / G1 stages between the *F. carica* treated and non-treated cells in MG-63. In HT-29, although an arrest in the S phase was detected ($p<0.007$).



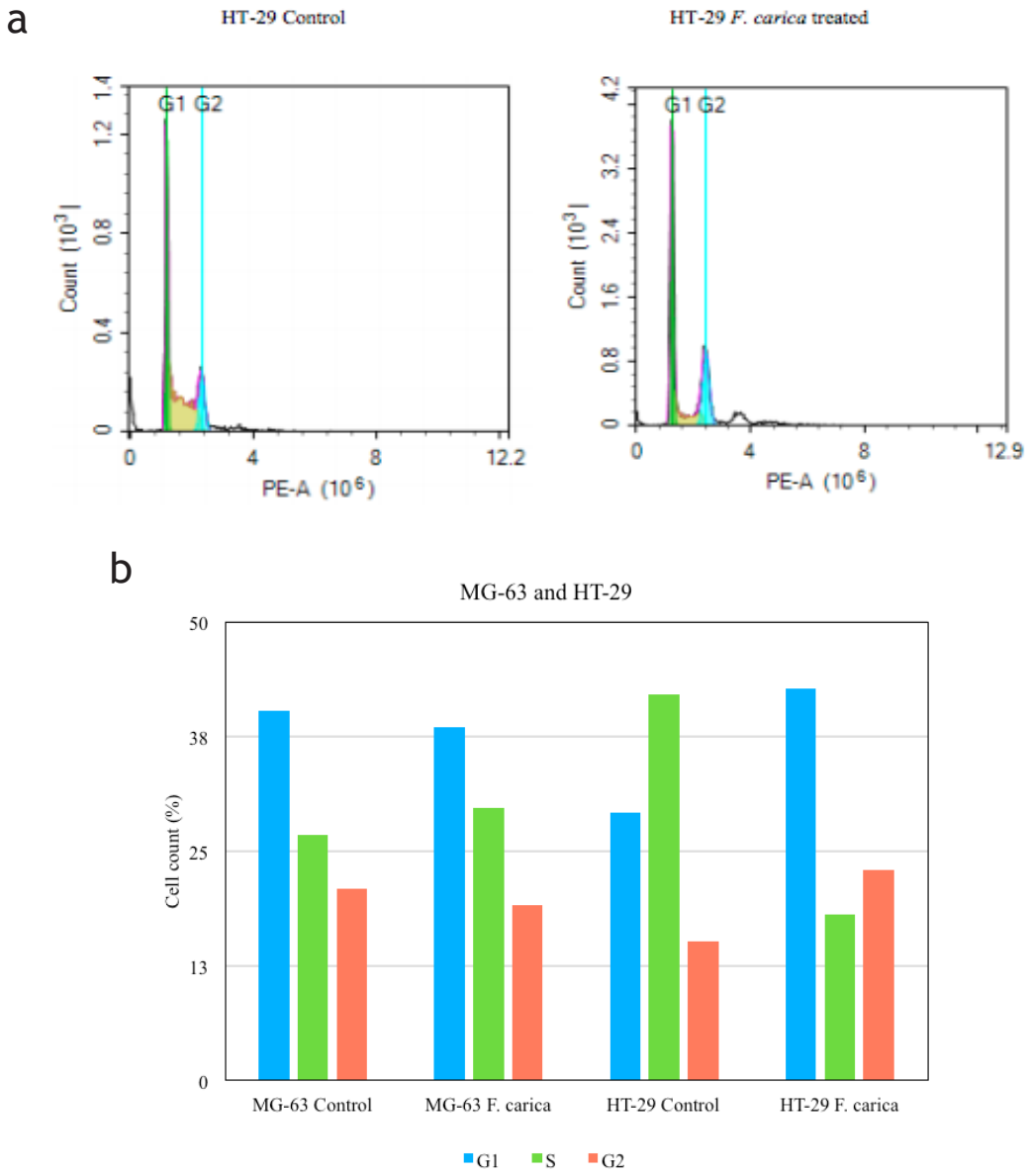


Figure 5. (a Cells were incubated with *F. carica* extract according to IC_{50} dose and time, stained with propidium iodide, and cell number in different phases of the cell cycle analyzed by flow cytometry. (b) Cell percentages of MG-63 and HT-29 relative to control cell line.
*Their values are different from the control groups ($p < 0.05$).

F. carica extracts induced apoptosis in MG-63 and HT-29 cell lines but in normal hFOB1.19 cells

In order to detect DNA fragmentation and loss of mitochondrial membrane, cells treated with *F. carica* extract were fluorescence stained by TUNEL assay. Increased fluorescence signal is present in MG-63 and HT-29, but not in hFOB1.19 (Figure 6).

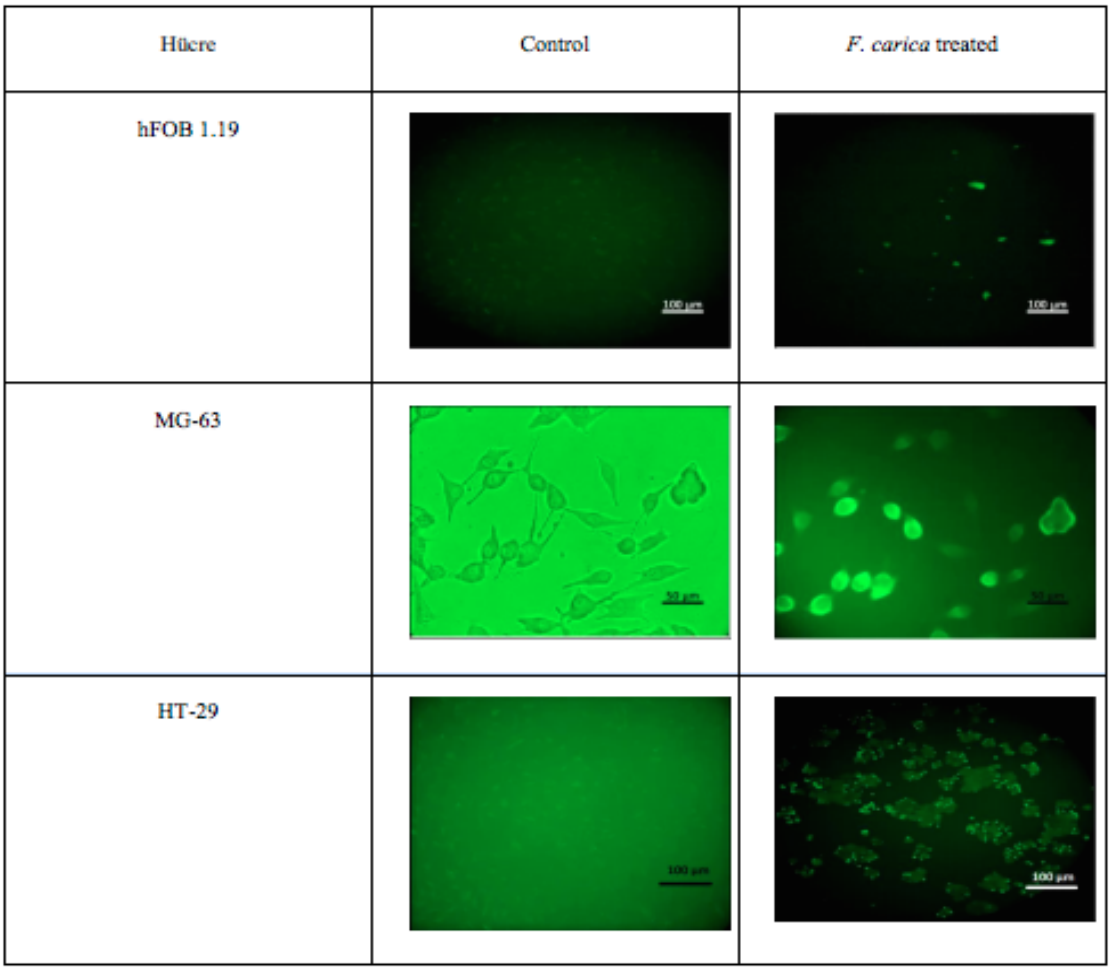


Figure 6. Microscopic detection of apoptosis using the TUNEL test in cells untreated and treated with *F. carica* extract.
TUNEL assay was performed after *F. carica* was administered according to the IC_{50} dose and time for hFOB 1.19, MG-63 and HT-29. Cells were seeded on chamber slides at a density of 4000 cells/cm^2. Excitation wavelength was studied under fluorescence microscopy at 450-500nm and scanning wavelength at 515-565nm. magnifciant science cular 10x (hFOB 1.19-HT-29), 20x (MG-63).

The formation of apoptosis was further confirmed using the Annexin-V and Dead Cell Assay

Apoptotic analysis of control and cancer cell lines was carried out by evaluating early stage, total apoptosis and late apoptosis. A significant reduction in cell survival was observed in both MG-63 and HT-29 cancer cell lines. However, the HT-29 cell line was observed to undergo mainly early-stage apoptosis, while late-stage apoptosis was observed in the MG-63 cell line. Interestingly, no significant change was observed in the hFOB 1.1.9 cell population in terms of dead cell analysis. The effects of *F. carica* extract on the growth of two cancer cell lines are shown in Figure 7.

The dramatic reduction in the number of viable cells in MG-63 and HT-29 cancer cell lines compared to normal cells was determined by the fold change. While a three-fold increase in early and total apoptosis were detected in MG-63 cells, the increase in early and total apoptosis for HT-29 cells is 12 and 1.5 folds, respectively. Mortality rate in MG-63 cell line was 40% higher than control cell lines ($p < 0.03$).

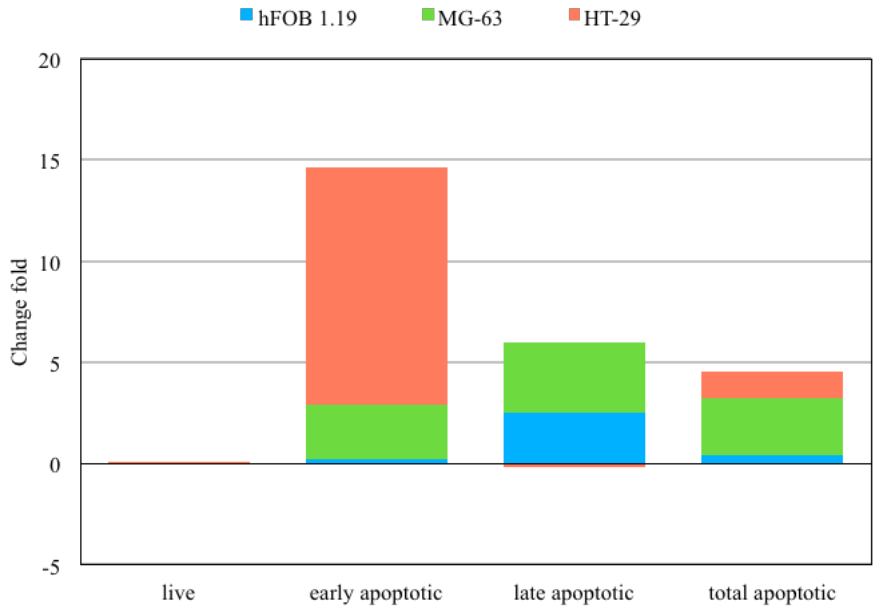


Figure 7. Determination of percentage of the apoptotic cells by Muse™ Annexin-V&Dead Cell Kit on the Muse cell analyzer. Annexin-V analysis was performed at a starting density of 1×10^6 cells were seeded onto regular plates. After treated with extract of *F. carica* was administered according to the IC_{50} dose and time for hFOB 1.19, MG-63 and HT-29. The bar graph shows the distribution of viable, early apoptotic, late apoptotic and total apoptotic cells by calculating the fold change of cells treated and untreated with *F. carica*.

The IC_{50} dose and time interval were determined with MTT assay was accepted as the treatment protocol for the further analyses in the study. According to MTT analysis, the optimal activity of *F. carica* in MG-63 and HT-29 cell lines was determined in 48 h at 1: 100 dilution and 24 h at 1:10 dilution, respectively. In addition, the cell viability did not drop below 50% in the hFOB 1.19 cell line at any dose and time interval, indicating that *F. carica* was not cytotoxic to normal cells.

DISCUSSION

AIn cancer treatment, it is a necessity to develop new therapeutic approaches to reduce drug costs, prevent high toxicity, and resistance to multiple drug combinations (14). Although studies have been conducted on the cytotoxic effects of *F. carica* extract on MCF7, HepG2, U2OS, MDA-MB-231 and T98G, U-138MG and U-87MG cell lines, its mechanism of action on cancer cell lines has not yet been fully explained (12, 15-17). Therefore, the anti-cancer potential of *F. carica* extract encouraged us to study its cytotoxic and apoptotic effects on the bone cancer cell line MG-63 and the colon cancer cell line HT-29.

Within the scope of the study, for the characterization of protocatechuic acid, the first standardization was carried out by HPLC method. According to HPLC analysis, *F. carica* used in this study contained $8.17063x10^{-1}$ mg/L protocathecuic acid which has been identified as an anti-cancer agent on various cancer cell lines (22). Thus, we think that protocathecuic acid may be partially responsible for the apoptosis inducing property of *F. carica* on MG-63 and HT-29 cells.

To analyze the potential anti-cancer activity of *F. carica* in 2 different cancer cell lines together with healthy cell line, we first evaluated the cytotoxic effect(s) of *F. carica* on MG-63, HT-29 and hFOB 1.19 cell lines using MTT assay. According to MTT analysis, optimal activity of *F. carica* in MG-63 and HT-29 cell lines was determined at 1:100 (33.3 mg/mL) dilution in 48 hours and 1:10 (333 mg/mL) dilution

in 24 hours, respectively. MTT assay showed that the two different cancer cell lines have different time points and dose. There is no study for IC_{50} level for MG-63 using *F. carica*, so far. There is only one study performed in HT-29, the IC_{50} level was found 261 µg/mL in whole fruit extract (23). On the other hand, IC50 dose in other cell lines which is not subjected to this study using whole *F. carica* extract was determined 13.4 mg/ml in Huh7it (24), 2.42 mg/mL in A549, 0.65 mg/mL in MCF-7, 36.02 mg/mL in HepG2 (25).

In addition, cell viability did not lead to decrease below 50% at any dose and time points in the hFOB 1.19 cell line. Future studies are needed to elucidate whether the difference in IC_{50} dose among cancer cell lines in previous studies is related to the origin of cell (MG-63 is bone originated, HT-29 is epithelial tissue). In the later stages of the study, *F. carica* was applied to the cells according to the determined IC_{50} doses and time points. A previous study has shown that the intrinsic cell death pathway by *F. carica* can be triggered as mitochondrial cytochrome C migrates into the cytoplasm (26). This situation has revealed that the necessity of clarifying the mechanism by which cell death pathways are activated.

An earlier study has shown that LDH leakage occurs only when cell death-related reactive oxygen species responsible for mitochondria damage. On the contrary, the MTT assay shows inhibition of cell growth at lower anti-tumor agent concentrations than the LDH assay. That is, the reduction of MTT results in cell death (27). In order to analyze better cytotoxicity in this respect, we measured the LDH activity of the MG-63, HT-29 and hFOB 1.19 cell lines.

The LDH analysis is a test for determining the cytotoxicity of an anticancer agent on cancer cells by measuring the level of lactate dehydrogenase. LDH, a cytoplasmic enzyme found in all cells is a marker for cell membrane damage, apopto-sis or necrosis (28). When the cells are exposed to toxic substances, the membrane structure is disrupted and LDH leaks out of the cells. Thus, the activity of intracellular enzymes released as a result of membrane change can be measured quantitatively with the LDH method (29). Bradford assay is used to measure the amount of released protein. In our study, the amount of enzyme per unit protein was determined. *F. carica* extract was applied at three different time points and in five different doses. Similar results provided as in MTT assay, LDH level was found low due to decreasing living cell numbers. LDH/Bradford ratio in all cells increased in direct proportion with dose and time compared to control cells, even though the increase in hFOB 1.19 relative to cancer cells was very small. The increase in enzyme activity for MG-63 is more aggressive. Since this linear increase in the amount of LDH was in parallel to the MTT result, this strengthened the idea that the cell's membrane structure was disrupted and the cell went into death. So far, there is no published data other than this study on lactate dehydrogenase enzyme activity in *F. carica* anticancer studies. These results showed that *F. carica* has a cytotoxic effect on cancer cell lines.

Insensitivity to growth inhibitors is one of the hallmarks of cancer survival strategy (26). This situation leads to the regulation of the cell cycle in cancer development. Flow cytometry method used for cell cycle analysis provides us quantitative measurement of fluorescence intensity. There is evidence that the *F. carica* inhibits the cell cycle during S phase in breast cancer cell line MDA-MB-231 (17). When MG-63 cells exposed to *F. carica*, there was no change in at any cell cycle stage. However, an arrest was determined in S phase for HT-29 cell line. Although this data strengthens the hypothesis that *F. carica* affects signals that inhibit/control the growth of tumor cells, additional studies are needed to confirm the findings.

Morphological manifestations of apoptosis are membrane blebbing (early stage), cell shrinkage, chromatin condensation and nucleosomal DNA fragmentation (late stage), formation of small vesicles and apoptotic bodies. In particular, the phosphatidylserine flip-flop from the plasma membrane to the outside of the cell is the signal detected by macrophages surrounding apoptotic cells

(18-21). In this study, we applied TUNEL method to observe the release of phosphotidyl-serine from the cell membrane into the cytoplasm and the Annexin V experiment to observe nuclear disintegration.

The TUNEL method has been used extensively over the past two decades to evaluate cell death in tissues. In fact, most dead cells stain positive for TUNEL since most programmed cell deaths cause double strand breaks (30). It has been proven that *F. carica* extract causes DNA fragmentation in the brain tumor cell line GBM (15). Within the scope of our study, TUNEL method was applied to demonstrate DNA fragmentation in control and cancer cell lines to determine whether it would have the same effect or not. As a result of the TUNEL study, fluorescent staining and apoptotic morphology was detected in cancer cell lines with apoptosis, but no staining was observed in control cells. TUNEL positivity is found in apoptotic cells, but contrary to popular belief, TUNEL positivity is not limited to apoptosis, regulated necrosis cells are also TUNEL positive (31). From this point of view, additional methods are required to elucidate the effect of *F. carica* on cell death mechanism for cancer cells.

Therefore, we further confirmed apoptosis using Annexin-V and the Dead Cell Assay. Apoptosis marker, annexin V can bind the membrane phospholipids which are released after the loss of membrane integrity (30). Apoptotic analysis in control and cancer cell lines was performed by evaluating early stage and total apoptosis since they may also show late stage necrotic apoptosis. When compared, we detected that cancer cell lines HT-29 underwent higher apoptosis in the early period, while the cell death ratio in MG-63 was higher in the late stage, raising the question of which death pathways were activated in the cells. In addition to, these results support the view that *F. carica* induced apoptosis in anti-cancer studies on the liver cancer cell line Huh7it (24).

This study indicated that *F. carica* leads HT-29 and MG-63 cells to apoptosis, with the formation of DNA breaks, reduction of mitochondrial activity, loss of cell membrane and Annexin V release, and causes cytotoxicity and cell cycle arrest. On the other hand, during the process of cell death, multiple mechanisms can be triggered reciprocally to cause several types of cell death to occur simultaneously. While there are multiple signaling pathways that control different types of cell death, these pathways can concurrently be linked, activated, and operated in parallel. Therefore, additional laboratory studies are needed to elucidate the anti-cancer activity of *F. carica*. Also, in the light of previous studies, protocathecuic acid may be partially responsible for the ability of *F. carica* to induce apoptosis in cancer cells. Furthermore, in future studies, providing the data obtained by detailed examination of all components in the *F. carica* extract; we will focus on other prospective candidate molecules.

In conclusion, this study demonstrates the anticancer effects of *F. carica* extract on MG-63 bone and HT-29 colon cancer cell lines. The data showed that while *F. carica* did not have a cytotoxic effect in normal cells, it had a cytotoxic effect in cancer cells, activated cell death pathways, arrested the cell cycle, impaired membrane integrity and caused DNA breaks. As a result, this study demonstrated the anticancer effect of *F. carica* on MG-63 and HT-29 cells and thus could be a potential resource for developing anti-cancer medicine and shed light on future research. More detailed studies are needed on cell death pathways using *F. carica* extract.

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ETHICS COMMITTEE APPROVAL

* This study does not require Ethics Committee Approval.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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