Research Article

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The cytotoxic and apoptotic effects of *Ferulago* W. Koch extracts on various cancer cell lines

[Ferulago W. Koch Türlerinin Çeşitli Kanser Hücre Hatlarında Sitotoksik ve Apoptotik Etkinlikleri]

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Abstract

Background: The studies investigating the anticancer activities of natural products have accelerated to produce new solutions in the face of increasing cancer cases. Various *Ferulago* species are reported to exhibit antioxidant, antiulcer and antimicrobial activities.
Objective: This study aimed to evaluate the cytotoxic and apoptotic activities of ethanolic extracts of roots of five *Ferulago* species on various human cancer cell lines.
Material and methods: HPLC analyses were performed by HP Agilent 1,100. The cytotoxicity were determined by MTT assay. The cell cycle arrest and Annexin V binding analyses were performed by Muse Cell Analyzer (Millipore).
Results: All examined species except *F. setifolia* inhibited cell viability in PC3 and SW480 cells at 0.01 mg/mL and higher concentrations ($p<0.05$). *Ferulago* species inhibited cell cycle at different stages for treated cell lines. The ethanolic extracts of *Ferulago* species also increased Annexin V binding significantly, resulted in apoptosis ($p<0.05\%$). In this context, *F. syriaca* showed the highest apoptotic activity in MCF-7 cells by increasing the apoptotic cell population to 23.54 ± 2.10% ($p<0.0001$).
Conclusion: The findings of present study have shown that *Ferulago* species included in the study have potent anticancer effects and this work have the potential to result in further studies.

Keywords: antiproliferative; apiaceae; apoptosis; cell cycle; Ferulago sp; medicinal plant.

Öz

Amaç: Doğal ürünlerin antikanser aktivitelerini araştıran çalışmalar, artan kanser vakaları karşısında yeni çözümler üretmek için giderek hız kazanmıştır. Çeşitli Ferulago türlerinin antioksidan, antiülser ve antimikrobiyal aktiviteler sergilediği bildirilmiştir. Bu çalışmada, beş Ferulago türünün köklerinin etanolik ekstraktlarının çeşitli insan kanser hücre hatları üzerindeki sitotoksik ve apoptotik aktivitelerinin değerlendirilmesi amaçlanmıştır.
Gereç ve Yöntem: HPLC analizleri, HP Agilent 1,100 ile yapıldı. Sitotoksisite, MTT testi ile belirlendi. Hücre döngüsü tutulumu ve Annexin V bağlanma analizleri, Muse Cell Analyzer (Millipore) ile gerçekleştirildi.
Bulgular: Elde edilen bulgulara göre, *F. setifolia* dışında incelenen tüm türler, PC3 ve SW480 hücrelerinde 0,01 mg/mL ve daha yüksek konsantrasyonlarda hücre canlılığını inhibe etmiştir ($p<0,05$). Ferulago türlerinin, ekstre uygulanan hücre hatları için farklı aşamalarda hücre döngüsünü inhibe ettiği gözlenmiştir. Ferulago türlerinin etanolik ekstreleri, Annexin V bağlanmasını önemli ölçüde artırmış ve apoptozu indüklediği belirlenmiştir ($p<\%0,05$).

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Bu kapsamda, *F. syriaca*'nın, apoptotik hücre popülasyonunu % 23.54 ± 2.10'a çıkararak MCF-7 hücrelerinde en yüksek apoptotik aktiviteyi gösterdiği belirlenmiştir (p<0,0001).

Sonuç: Bu çalışmanın bulguları, çalışmaya dahil edilen Ferulago türlerinin güçlü antikanser etkilere sahip olduğunu ve bu çalışmanın daha ileri çalışmalara dayanak oluşturma potansiyeline sahip olduğunu göstermiştir.

Anahtar kelimeler: Antiproliferatif; Apiaceae; Apoptoz; Hücre döngüsü; Ferulago sp; Tıbbi bitki.

Introduction

Cancer is a common and important reason of morbidity and mortality throughout the world [1]. Radiotherapy and chemotherapy are conventional therapies that can be used to fight cancerous cells, however while suppressing different tumor cell lines, they also have significant side effects which led scientists to seek for alternative therapies. Thus, different sources have started to be examined for their anti-tumoral activities in the search for finding effective treatments with low side effect profiles [2].

Plants are known to contain many phytochemicals, each of which can have different biological effects. These phytochemicals exert their effects via various pathways and are considered to be promising anti-cancer metabolites [2]. In recent years, thousands of plant species have been tested in search as novel candidates against cancer [3].

Among these phytochemicals, coumarins are attracting interest due to their important and diverse biological activities. High plants, bacteria and fungi are known to yield more than 13,000 coumarins until now and in respect to high plants, they are known to be present over nearly 30 different families; Apiaceae family is one of them and it is also among the most important coumarin yielding families [4]. Naturally occurring coumarins are known to possess a wide range of activities e.g. antioxidant, anti-inflammatory, anticancer, monoamine oxidase-B inhibitory, antimicrobial, anti-allergic, hepatoprotective, antithrombotic, and antiviral activities [5–9]. They also have prominent anticancer properties with low adverse effects based on the functional groups in the original structure. They can effect different cellular pathways [10], and the induction of apoptosis in malignant cells is an important issue that researchers throughout the world are focusing on [3]. Apoptosis is an important process in the fight with cancer since it is actually a mode of self-cannibalism involving individual cells and in contrast to necrosis, it does not result in inflammation in the neighboring cells [11].

When we search the literature, we can see that several *Ferulago* W. Koch species were tested on different tumor cell lines and were shown to exert anticancer activity. In addition to these studies, which were carried out on extracts prepared from the plant species with different solvents, some coumarin derivatives were also isolated and tested, and promising results were obtained. *Ferulago angulata* (Schlecht) Boiss. has been studied extensively for its effect on cancer cell lines. For example in a study by Amirghofran et al. [12], extract prepared from aerial parts of the plant demonstrated anti-cancer effect on K562 leukemia cell line and inhibited the proliferation of Jurkat cells. In another study on *F. angulata* species, extract of the plant was shown to suppress proliferation of three leukemia and lymphoma tumor cell lines in a dose and time dependent manner and during this process peripheral mononuclear cells were not significantly affected [13].

Extracts prepared from other *Ferulago* species were also studied in this respect. Hexane and ethyl acetate fractions of aerial parts of another *Ferulago* species, *F. carduchorum* Boiss and Haussk yielded cytotoxic activity toward cell lines due to coumarins and phytosteroids [14]. *F. confusa* Velen. was also found to be cytotoxic in a study performed on brine shrimp. Though this study did not focus on anti-cancer activity, the species still might have anti-cancer effect if studies directed to this effect were performed [15]. In another study conducted on the aqueous extracts prepared from the roots and aerial parts of *Ferulago mughlae* Peşmen, these extracts were found to inhibit cell proliferation when tested on SW480 colorectal carcinoma cells [16].

In the light of these aforementioned studies performed with various extracts obtained from different *Ferulago* species, we decided to investigate the cytotoxic activity of the root extracts of some *Ferulago* species (*F. syriaca*, *F. longistylis*, *F. isaurica*, *F. setifolia* and *F. cassia*) growing naturally in Turkey against some different cancer cell lines as a preliminary study. It is also worth mentioning that two of these species (*F. isaurica* and *F. longistylis*) are endemic for Turkey.

Materials and methods

Plant material

Collection localities of the plant materials are given in Supplementary Table 1. Plant species were identified by H. Duman from Gazi

University Faculty of Science, Department of Biology who is also one of the authors and voucher specimens are kept in AEF Herbarium (Herbarium of Ankara University Faculty of Pharmacy).

Extraction procedure

Thirty gram of roots of each species were grounded and placed in separate vessels. 200 mL of 80% ethanol were added to each vessel and macerated in a shaker for 48 h at 140 rpm. At the end of this period, extracts were filtered and the residuum of all extracts was subjected to the same process for six times more. Filtered extracts were combined and the solvent was evaporated with the help of a rotary evaporator (Heidolph Hei-Vap Advantage, Germany) at a temperature not exceeding 45 °C.

HPLC studies

Ethanol extracts and the standard substances felamedin and prantschimgin, both were isolated by Erdurak-Kilic et al. [17] were analyzed by HP Agilent 1,100 (Germany). Felamedin and prantschimgin standards were weighed with an analytical scale and solutions were prepared with a concentration of 1 mg/mL and calibrations were performed with these stock solutions. The HPLC chromatograms of felamedin and prantschimgin standards were given in Supplementary Figure 1.

Cell culture studies

A549 human lung carcinoma, PC3 human prostate carcinoma, MCF-7 human breast carcinoma and SW480 human colon carcinoma cell lines were purchased from the American Type Cell Culture Collection (ATCC, Germany). The cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (Lonza, Germany) in 5% CO_2 incubator at 37 °C. The cells were treated with different concentration of extracts ranged between 0.01 and 1 mg/mL for 24 h under the same culture conditions. The stock solutions of extracts were dissolved in DMSO (dimethylsulfoxide) (Sigma, Germany), and the final concentration of this solvent was kept constant at 0.01%. The non-treated cells were used as control in cell assays.

Cell proliferation assay

The antiproliferative effects of extracts on cell lines were evaluated by MTT [3-(4,5-dimethylthiazol-2yl)-2,4-diphenyltetrazolium bromide] assay, which is a reliable and basic method for preliminary evaluation of anti-cancer agents [18]. The cells were seeded in 96-well plates at density of 9×10^3 cells/well and incubated for 24 h at 37 °C, then treated with 0.01, 0.025, 0.05, 0.1 and 1 mg/mL of the extracts for 24 h. The dose administered was increased to 10 mg/mL in the MCF-7 cell line alone so that the appropriate IC_{50} value could be calculated. Following incubation, the cells were treated with MTT solution (5 mg/mL, dissolved in cell medium) and incubated for 2 h. The formazan crystals were dissolved in DMSO and the absorbance was recorded at 540 nm by spectrophotometer (Thermo, Germany). Results are expressed as the mean ± standard deviation (SD) of three independent experiments. The differences are *$p<0.05$ and #$p<0.0001$ compared to control. The IC_{50} value (the half maximal inhibitory concentration) was calculated by cell proliferation results through linear regression analysis through GraphPad Prism 6.0 version (GraphPad Software Inc.).

Cell cycle analysis

Muse Cell Cycle Assay Kit (Millipore, Germany) were used to determine the effects of extracts on cell cycle arrest. The cells were plated on 12-well plates at a density of 2×10^5 cells/well and incubated for 24 h at 37 °C, then treated with ethanol extracts of roots of *Ferulago* species at IC_{50} concentrations, which previously determined by MTT assay. Following 24 h incubation, the cells were harvested and then fixed with 70% ethanol at 4 °C for 4 h. Cell pellets were then collected by centrifugation at 400×*g* for 10 min and incubated with assay solution for 30 min. The Muse Cell Analyzer (Millipore, Germany) has been used to detect three phases of the cell cycle including G0/G1, S and G2/M.

Annexin V binding assay

The cells were plated into 6-well plates at a density of 1×10^6 cells per well and incubated for 24 h. The cells were then treated with ethanol extracts of roots of *Ferulago* species at IC_{50} concentrations for 24 h. Nontreated cells were defined as control group. Following incubation, the cells were harvested, centrifuged and resuspended in DMEM medium. Annexin V assay kit (Millipore, Germany) was performed according to the manufacturer's instructions. Briefly, 100 µL of reagent including Annexin V and 7-aminoactinomycin D (7-AAD), a dead cell marker, was added to 100 µL of cell suspension and incubated for 20 min at room temperature. Then, the apoptotic cell population was detected by Muse Cell Analyzer (Millipore). Four populations of cells can be distinguished in this assay: non-apoptotic cells: Annexin V (–) and 7-AAD (–); early apoptotic cells: Annexin V (+) and 7-AAD (–); late stage apoptotic and dead cells: Annexin V (+) and 7-AAD (+); mostly nuclear debris: Annexin V (–) and 7-AAD (+).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 version (GraphPad Software Inc.). Data obtained from the cell culture experiments were expressed as mean ± SD, and a one-way ANOVA test was applied for multiple comparisons. The IC_{50} values were calculated by linear regression analysis within the same analysis software.

Table 1: Extract yields of *Ferulago* species.

Species	Extract amount obtained, g	% Yield [(g extract/g dry root) × 100]
F. cassia	5.8353	19.45
F. isaurica	6.1352	20.45
F. syriaca	4.9117	16.37
F. longistylis	7.2063	24.02
F. setifolia	4.9120	16.37

Results

The ethanolic extracts of roots for each species were prepared as described in methods section. The extract yields are given in Table 1.

HPLC analysis

Only one of the chromatograms belonging to *F. longistylis* is given in Supplementary Figure 1 in order not to take up too much space. The presence of the peaks for both standard substances were confirmed with two methods: First external standard method was used: a quantity of the standards were individually added to the extract and the increase in peaks areas were observed. Then the signals that we have obtained for the standard substances were overlaid with the signals obtained from the extracts and as a result it could be concluded that both felamedin and prantschimgin were present in all of the extracts. The calculations for calibration of standards were given in Table 2.

The felamedin and prantschingin quantities of the extracts were given in Table 3. As it can be seen from the table, the highest felamedin and prantschimgin concentrations are found in the extract of *F. longistylis* species.

Table 3: Felamedin and prantschingin quatities of the extracts.

Species	Measured concentration, mg/mL	
	Felamedin	Prantschimgin
F. cassia	0.5133	0.5015
F. isaurica	0.1001	0.5015
F. longistylis	1.6660[a]	0.6045[a]
F. setifolia	0.3623	0.2344
F. syriaca	0.4697	0.1459

[a]Specifies highest quantities of corresponding coumarin derivatives.

Cell proliferation

The antiproliferative effects of extracts of different *Ferulago* species were evaluated by MTT test and the results were given in Figure 1. In A549 cells, the cell viability was decreased significantly at 0.1–1 mg/mL concentrations (Figure 1A). In *F. longistylis and F. syriaca* treated groups, the viable cell amount significantly decreased to 25.37 ± 3.28% and 24.55 ± 2.31%, respectively at 1 mg/mL concentration when compared to control ($p<0.0001$). The IC_{50} values of these two species were calculated as 0.629 and 0.646 mg/mL, respectively (Table 4). On the other hand, *Ferulago* species were also cytotoxic to MCF-7 cells since the cell viability significantly decreased at 0.025 mg/mL and higher concentrations in *F. cassia* and *F. longistylis* treated groups (Figure 1B) when compared to control ($p<0.05$). The cytotoxicity results of *Ferulago* species on PC3 cells were shown in Figure 1C. The cell viability significantly decreased at all treated concentrations in *F. cassia, F. longistylis, F. isaurica* and *F. syriaca* treated groups and *F. longistylis* was determined as the most effective species with 0.393 mg/mL IC_{50} value (Table 4) in this cell line. Figure 1D represents the MTT results of extracts on SW480 colon carcinoma cells. The ethanolic extract of *F. isaurica* exhibited the most cytotoxic effect on SW480 cells through decreasing viable cell amount to 27.11 ± 0.46% ($p<0.001$) with 0.267 mg/mL IC_{50} value (Table 4).

Cell cycle assay

The significant findings from antiproliferative activity studies, led us to research the effects of extracts on cell cycle arrest. In this context, the results of cell cycle analyses of four different cell lines were given in as a bar graph in Figure 2 and the cell cycle plots of extracts with the lowest IC_{50} values for each cell line were also represented in Figure 3. In A549 cells, all of the ethanolic extracts of roots of *Ferulago* species induced a significant cell cycle arrest at G0/G1 phase (Figure 2A). In *F. longistylis* treated group, the cell population % at G0/G1 phase significantly increased to 63.90 ± 3.70%, while it was 46.20 ± 1.30 ($p<0.0001$) in control group (Figure 3). In MCF-7 treated group, the extracts of *Ferulago* species significantly increased the cell population % at G2/M phase of cell cycle (Figure 2B) and the ethanolic extract of *F. setifolia*, with 2.259 mg/mL IC_{50} value, increased cell

Table 2: Calculations for calibration equations of felamedin and prantschimgin.

	Slope	Intersection	Regression coefficient (R^2)	LOD	LOQ
Felamedin	17430	2248.9	0.9684	0.0022	0.0069
Prantschimgin	17673	2949.9	0.966	0.0381	0.0177

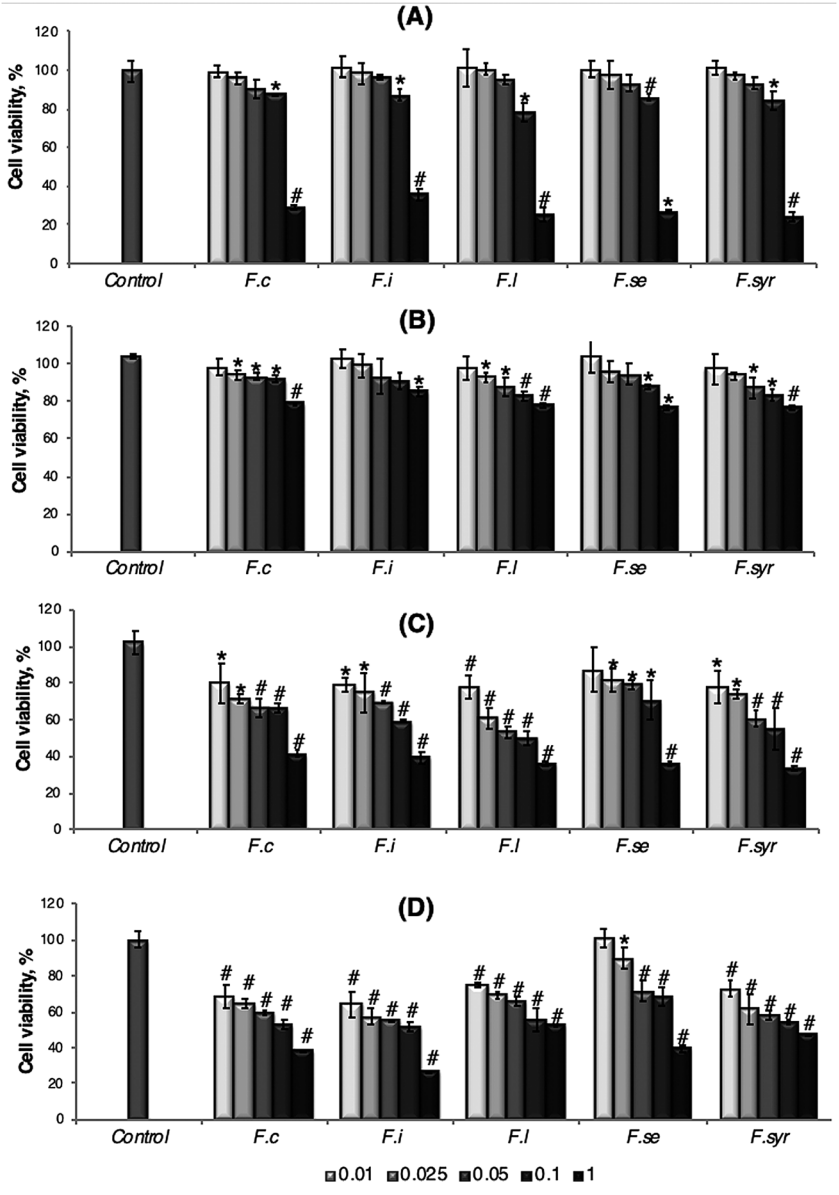


Figure 1: The effect of the ethanolic extracts of *Ferulago* species on cell proliferation of A549 (A), MCF-7 (B), PC3 (C) and SW480 (D) carcinoma cells. The cells were treated with root extracts (0.01, 0.025, 0.05, 0.1, 1 mg/mL) for 24 h and treated with MTT solution. Results are expressed as a percentage of viable cell amount. The nontreated cells were used as control. Each value represents the mean ± standard deviation from three independent experiments performed in triplicate (*$p<0.05$, #$p<0.0001$, compared to control). The abbreviations for F.c., *F. cassia*; F.i., *F. isaurica*; F.l., *F. longistylis*; F.s., *F. setifolia*; F.s., *F. syriaca*.

Table 4: IC_{50} values mg/mL of extracts for different cell lines.

Species	A549	MCF-7	PC3	SW480
F.cassia	0.683	2.682	0.718	0.507
F.isaurica	0.770	3.731	0.667	0.267
F.longistylis	0.629	2.813	0.393	1.111
F.setifolia	0.669	2.259	0.689	0.745
F.syriaca	0.646	2.695	0.511	0.772

population from 35.60 ± 2.5 to 61.90 ± 3.20% ($p<0.0001$) at G2/M phase, while cell population % decreased from 6.80 ± 0.50 to 5.10 ± 0.30% and from 56.90 ± 2.50 to 32.60 ± 2.30% at G0/G1 and S phases, respectively, when compared to control (Figure 3). The cell cycle analysis results for PC3 cells were similar to results of A549 cells (Figure 2C). The extract of *F. longistylis*, with 0.393 mg/mL IC50 value, induced cell cycle arrest at G0/G1 phase significantly when compared to control ($p<0.0001$), (Figures 2C, 3). In SW480 cells, the ethanolic extracts of *F. cassia and F. syriaca* induced cell cycle arrest at G2/M phase, whereas *F. isaurica*, which has the lowest IC_{50} value as 0.267 mg/mL on SW480 cells, inhibited cell proliferation through an arrest at S phase of cell cycle (Figures 2D, 3).

Apoptosis assay

The effects of ethanolic extracts of roots of *Ferulago* species on apoptosis of cancer cells were evaluated by Annexin

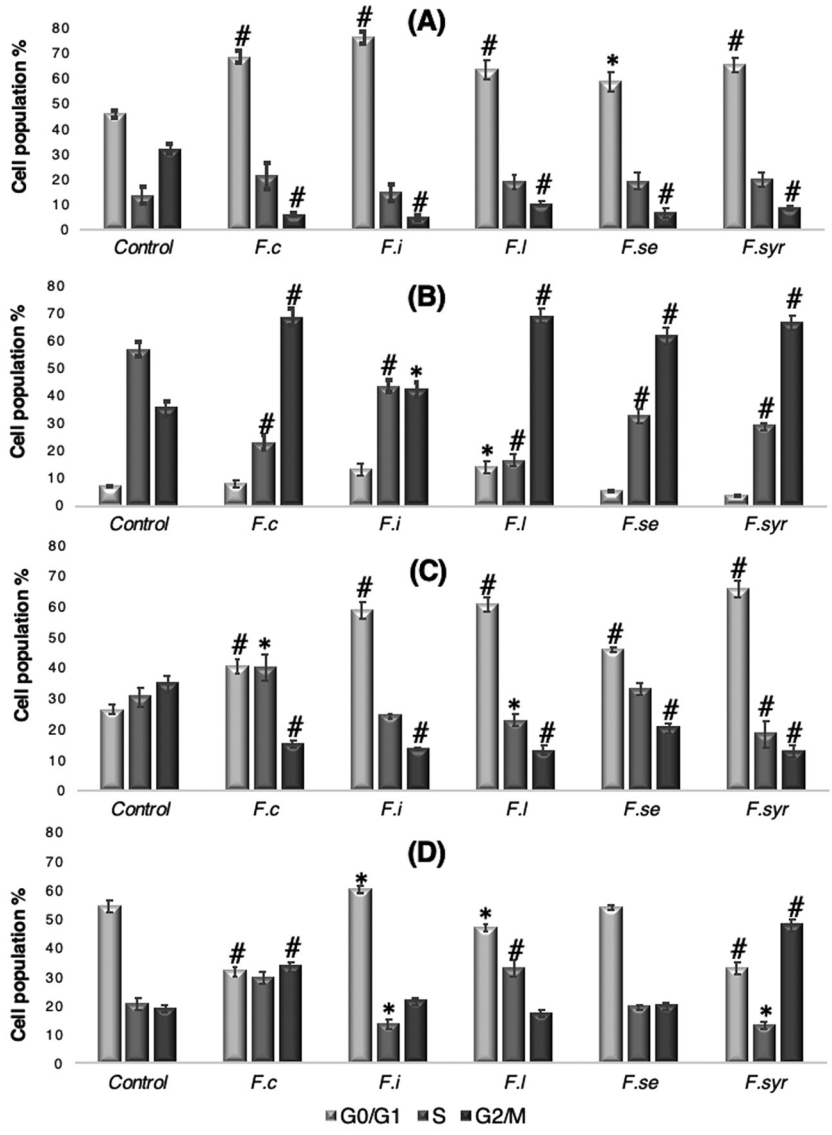


Figure 2: The results of cell cycle analysis. The A549 (A), MCF-7 (B), PC3 (C) and SW480 (D) cells were treated with ethanolic extracts of roots of *Ferulago* species at IC_{50} concentrations and cell cycle assay was performed. The cell population percent at different phases of the cell cycle, including G0/G1, S, and G2/M were detected by Muse Cell Analyzer. The results were given for three independent experiments. The differences are *$p<0.05$, #$p<0.0001$, compared to control.

V binding assay. This assay utilizes Annexin V to detect phosphotidiylserine on the external membrane of apoptotic cells. It is excluded from live, healthy cells, as well as early apoptotic cells. According to the results of annexin V binding assay, the cell population % corresponding to live, total apoptotic and dead cells were calculated and the results of annexin V binding effects of extracts were represented as bar graph in Figure 4. On the other hand, the plots for the species which were determined to have the most apoptotic activity among *Ferulago* species are shown in Figure 5. In A549 and SW480 cell lines, *F. cassia* showed the highest apoptotic activity (Figures 4A, D, 5), since the total apoptotic cell population significantly increased to 15.75 ± 1.64 and 21.60 ± 2.83%, while it was recorded as 5.55 ± 0.34 and 2.25 ± 0.24% in control groups of A549 and SW480 cell lines, respectively ($p<0.0001$). *F. syriaca* was determined as the most effective species on apoptosis of MCF-7 cells (Figures 4B, 5). The 23.54 ± 2.10% of cell population were determined as annexin V positive ($p<0.0001$). In PC3 cells, although all species decreased cell viability, the significant increase in apoptotic cell population were observed in *F. cassia*, *F. isaurica*, *F. longistylis* and *F. setifolia* treated groups (Figure 4C). Among these species, *F. setifolia* increased total apoptotic cell population % to 11.60 ± 1.49%, while it was 1.32 ± 0.21% in control group ($p<0.0001$) (Figure 5).

Discussion

In order to find novel agents for the treatment of cancer, which is one of the most important causes of death, studies

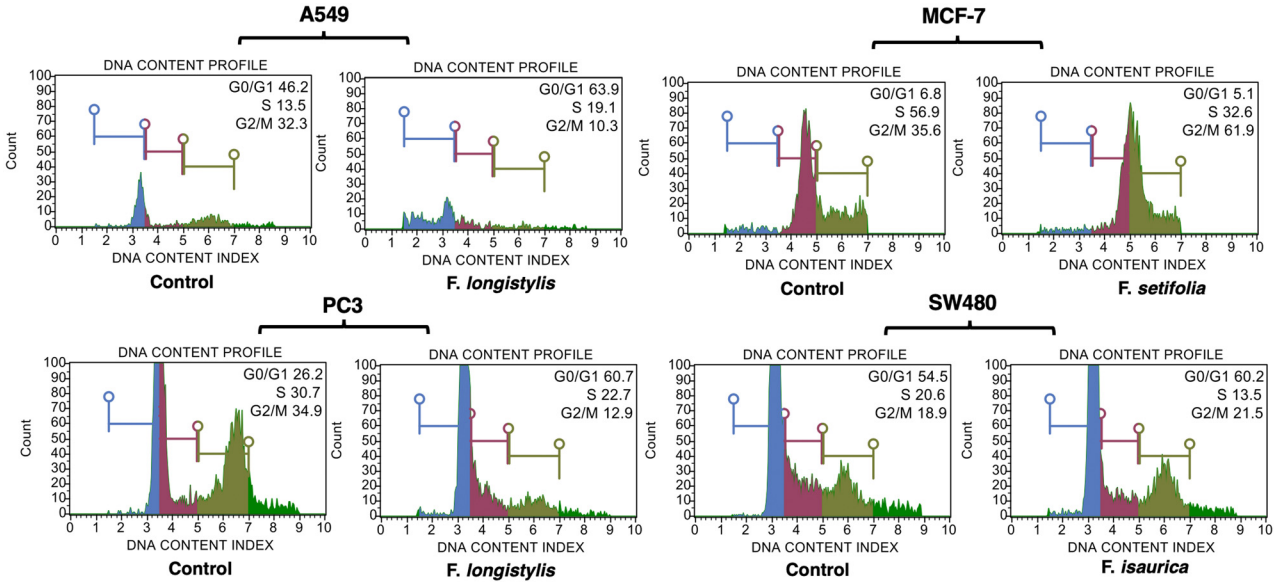


Figure 3: The cell cycle plots of various *Ferulago* species for cancer cell lines. The figure represents the plot graphs of *Ferulago* types which showed the lowest IC_{50} values in MTT analysis for each cell line. In this context, cell cycle plot graphs of *F. longistylis* for A549 and PC3 cell lines, *F. setifolia* for MCF-7 cells and *F. isaurica* for SW480 cells are given.

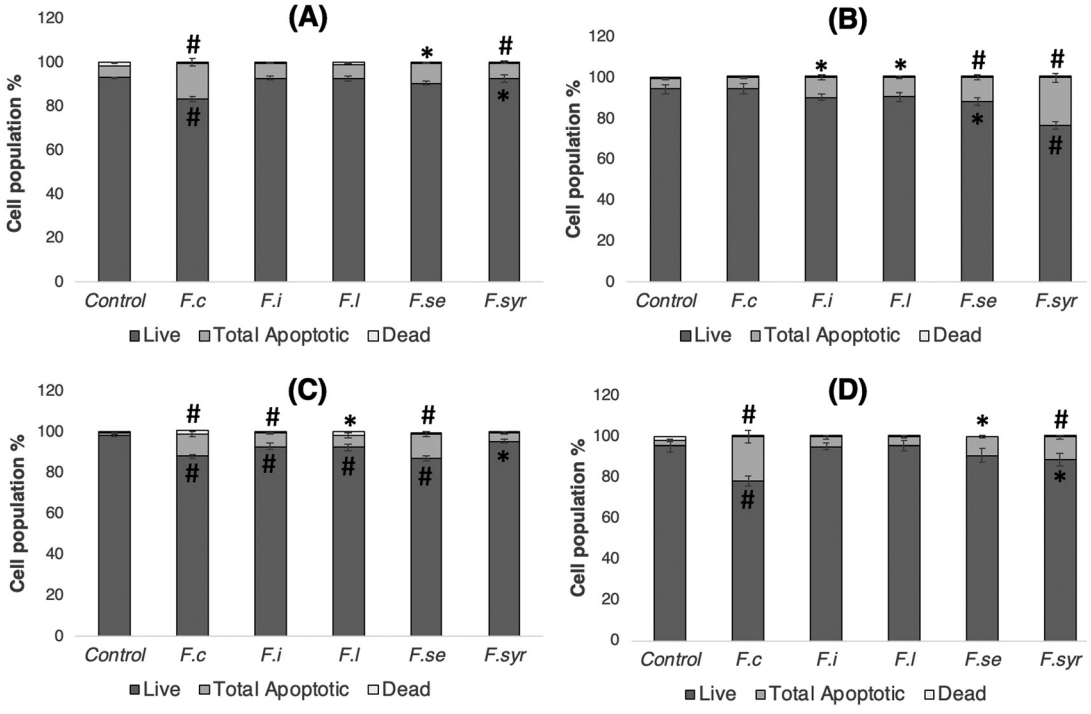


Figure 4: The results of the Annexin V binding assay. The The A549 (A), MCF-7 (B), PC3 (C) and SW480 (D) cells were treated with ethanolic extracts of roots of *Ferulago* species at IC_{50} concentrations and the apoptosis was detected by the Muse cell analyzer. Nontreated cells were used as control. The results were given for three independent experiments and the differences are *$p<0.05$, #$p<0.0001$ from control.

on the effectiveness of natural compounds have been of great interest [19]. Natural products of plant and animal origin have been used in medical applications for many years. Studies in recent years have shown that active natural compounds can be effective agents in various types of cancer and inflammation [20–24]. When all these data are

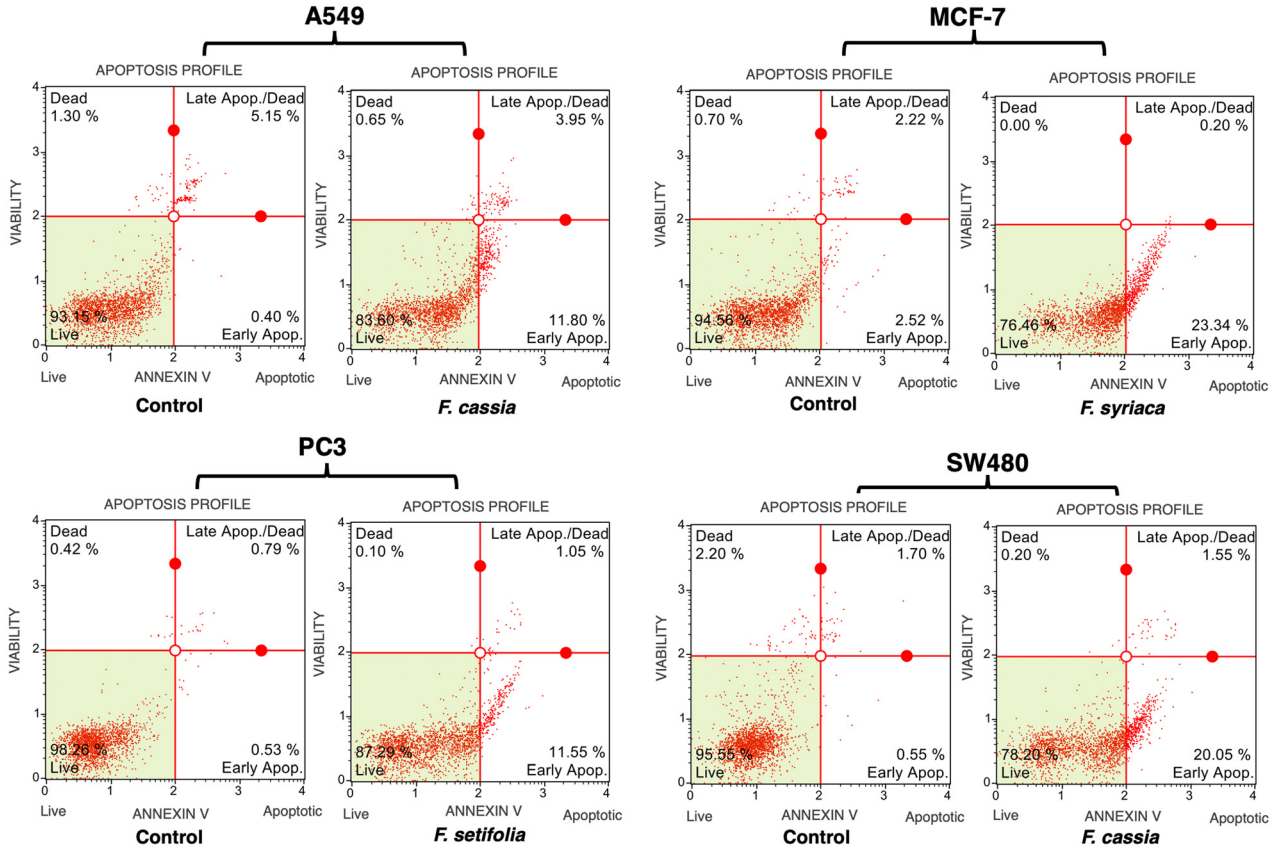


Figure 5: The annexin V binding assay plots of various *Ferulago* species for cancer cell lines. The figure represents the plot graphs of *Ferulago* types which showed the highest increase in apoptotic cell population for each cell line. In this context, annexin V binding assay plot graphs of *F. cassia* for A549 and SW480 cell lines, *F. syriaca* for MCF-7 cells and *F. setifolia* for PC3 cells are illustrated.

evaluated, the assessment of efficiency of crude extracts prior to demonstrating active compounds might be an important starting point in researches.

Although various activities of some *Ferulago* species have been identified by several biomedical studies, there is not still sufficient research in the literature about anticancer activities of all species. *Ferulago* genus has nearly 50 species throughout the world and approximately 35 taxa grow naturally in our country. Furthermore, nearly 18 of these taxa are endemic species for our country and this makes Turkey the gene center for the genus *Ferulago* (16). Therefore, in our current study, the cytotoxic and apoptotic effects of the extracts of various *Ferulago* species on different cancer cells were evaluated and it is also worth mentioning that two of these species were endemic species.

In literature there are some studies related with anticancer activities of *Ferulago* species. The cytotoxic efficiency of the essential oil and extract of different *Ferulago* species including *F. angulata*, *F. carduchorum* Boiss and Hausskn, *F. campestris* Grec. were investigated against different types of cancer cell lines and the results have demonstrated their significant cytotoxic effects [25–29]. It's been reported that some isolated compounds from *Ferulago* species including xanthotoxin, isoimperatorin and oxypeucedanin displayed significant cytotoxic activities against different cell lines [26]. It's reported that, leaf hexane extract of *F. angulata* induced apoptosis in MCF-7 cells (breast cancer) via a mitochondrial-dependent pathway and cell cycle arrest [30, 31]. In a study by Mirzaghaei et al. [2], ethanol extract of the leaves was found to inhibit sprouting, tube formation and migration capacities and therefore considered to possess anti-angiogenic compounds and concluded to be a promising species in therapies that focus on tumor anti-angionesis. The species was also found to be effective on HL-60 human promyelocytic leukemia cell line by inducing apoptosis [32]. Methanol extract of the aerial parts of the plant was tested on other cell lines, as well. For examples, the extract was studied on some other cell lines and was found to be active against B-cell lymphoma (Raji), human leukemic monocyte lymphoma (U937) and human acute myelocytic leukemia (KG-1A) probably via apoptosis-dependent pathway.

However, the extract had no effect on normal cell line HUVEC [33]. Even essential oil of the species was found to be effective against HEP-G2 human liver cancer cell line and T47D breast cancer cell line [29]. Although compound(s) that is(are) responsible for the activity are not determined at the present study, one of these coumarins could probably appear as the compound(s) which responsible for activity with the future planned bioactivity guided fractioning studies since various coumarins are known to possess anticancer effects and have been investigated extensively by many researcher around the world [21–24]. At present study, we evaluated the antiproliferative and apoptotic effects of ethanolic extracts of roots of some *Ferulago* species on different cancer cell lines. Our results indicated that the extracts could inhibit cell proliferation significantly, when compared to non-treated control cells. Among treated cell lines, MCF-7 human breast adenocarcinoma cells were determined as the most resistant to extracts, since the IC_{50} values were determined over 1 mg/mL, the maximum treated dose in cytotoxicity analysis. However, other cell lines were seemed more prone to cytotoxic effects of ethanolic extracts. On the other hand, this study has also demonstrated that the ethanolic extracts of roots have varied apoptosis inducible effects at their determined IC_{50} values against cancer cells. The results showed that, the effects of *F. setifolia* on cell cycle arrest and apoptosis were compatible in MCF-7 cells, which were also similar for *F. longistylis* in PC3 cells. Among treated species, *F. syriaca* has shown the most apoptotic effects through increasing total apoptotic cell amount from 4.74 ± 0.52 to $23.54 \pm 2.10\%$ in MCF-7 cells. This apoptotic effect was related with the decrease in cell viability through a cell cycle arrest at G2/M phase of cell cycle.

Standardization is an important issue in herbal medicine extracts. As it is well known, the content of active substances might differ due to many factors such as collection date, climatic conditions/seasonal differences, difference in the soil content, extraction method etc. even for the same population. Therefore, the extract that would be used for bioactivity studies has to be standardized over one compound at the least. Standardizations performed on more compounds would even be more reliable. Therefore, we standardized the extracts over two coumarin derivatives that are found to be present in many Ferulago species [4, 17, 34–44], and then we studied the biological activities of the extracts. That is to say, the studies were performed by using extract that contained these two coumarin derivatives (in mg/mL) as specified in Table 3. By this way, we tried to achieve reproducibility and reliability for future studies.

Furthermore, prantschimgin is reported to have anticancer effect [39, 44], we believe that these two compounds which are similar to each other in respect to structure, having close molecular weights, isolated from the plant as a mixture, and especially prantschimgin can be responsible for the cytotoxic effect that we have determined.

Conclusions

At present study, the cytotoxic and apoptotic activities of various *Ferulago* species on human cancer cell lines were evaluated. The findings revealed that the studied species showed significant cytotoxic activity on cancer cells. However, cell lines other than MCF-7 were found to be more sensitive to the extracts in question. The altered cytotoxic activities of *Ferulago* species on different cell lines were also supported by the finding of inhibitory activities of extracts on different stages of the cell cycle. Studies have also shown that all ethanolic extracts significantly induced cell apoptosis. However, *F. cassia* in A549 and SW480 cells and *F. syriaca* in MCF-7 cells were found to have the highest apoptotic activity. According to the results of this preliminary study, we may consider these extracts as promising candidates against treated cancer cell lines. To the best of our knowledge, the cytotoxic and apoptotic activities of roots of the *Ferulago* species mentioned here have been reported for the first time. Further studies are required to clarify the mechanisms underlying their cytotoxic effects and our research group is also would like to conduct studies in order to identify the active substance(s) responsible for anti-cancer effect in the future.

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