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Ferutinin content and cytotoxic effects of various *Ferula* L. species on prostate cancer (PC-3) cell line

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ABSTRACT: *Ferula* L. is represented by 23 taxa in Turkish flora. Most of the species are known as "Caksır" and roots are used as aphrodisiac in Anatolia. The aim of this study is to investigate the cytotoxic potential of roots of twelve different *Ferula* L. species distributing in Anatolia on prostate cancer cell line PC-3. Three extracts with different polarity were prepared from grounded roots of plants by ultrasonic solvent extraction. WST assay was performed to evaluate the cytotoxic effects of extracts. High cytotoxic activity was observed in *F. elaeochytris*, *F. drudeana* chloroform extracts and *F. szowitsiana* hexane extract (IC50: 3.21±0.24µg/ml, 5.92±0.53µg/ml, 8.03±0.51µg/ml respectively) whereas methanol extracts of all species had no cytotoxic activity. *F. tingitana* chloroform extract induced high proliferation in dose dependent manner. A daucane ester "ferutinin" content was evaluated among the extracts by UPLC and it is found in highest concentrations in *F. tenuissima* and *F. halophila* hexane extracts (167380 µg/g and 157160 µg/g respectively). Although there is not a direct relationship between ferutinin content and cytotoxicity on PC-3, *F. elaeochytris*, *F. drudeana*, *F. szowitsiana* and *F. tenuissima* can be good candidates for the further studies for finding lead phytochemical compounds for prostate cancer treatment.

KEYWORDS: *Ferula*, ferutinin, cytotoxic, PC-3, prostate cancer.

1. INTRODUCTION

The genus *Ferula* L. (Apiaceae) comprises about 180-185 species distributed from Asia, North Africa to North America[1] . It is represented by 23 taxon in flora of Turkey, 13 of which are endemic (% 62.3) and mainly distributed in Mediterranean, South-East and Inner Anatolia [2] .High endemism ratio indicates the position of this genus for Anatolia. The taxonomic and morphologic revision of *Ferula* L in Turkey is performed in 2005 [3]. *Ferula* species are erect, perennial plants growing up to 2-5 m tall. Roots are thick, woody, hard and fibrous. Basal cauline leaves are taxonomically important characters, 3-7 pinnate, segments differ filiform to setaceous. Inflorescence is paniculate-corymbose, petals are yellow [4,5].

Most of the species are known as "çakşır otu, cağşır and at kasnısı'' and used as aphrodisiac, tonic, antimicrobial, expectorant, in hemorrhoids and urinary diseases in Anatolian traditional medicine. Boiled leaves of *F. communis* L. *F. orientalis* L, *F. rigidula* DC. are consumed as food in east Anatolia. Roots of *F. elaeochytris* are used to increase the sexual performance in Adana, Hatay region[6]. Researches on cytotoxic and cancer preventing activities and hormonal effects of *Ferula* species have an increasing interest in recent years. Activities are generally related to the daucane sesquiterpenes and coumarin derivatives as the main metabolite groups of genus [7–13]. Especially ferutinin (a daucane type sesquiterpene ester) has been reported as a phytoestrogen acting as agonist for ER-α and agonist/antagonist for ER-β [14].

Prostate cancer is a life-threatening disease for male as it is the third most common cause of death from cancer among men [15]. New approaches are needed for the treatment of PC-3 because of the side effects and complications of current treatment options like chemo-, hormonal or radiotherapy [16,17]. The literature indicates that many phytochemicals or extracts of medicinal plants have positive effects against commonly to occur cancers such as prostate as compared with these therapies [18–21]. PC-3 cells are androgen-independent

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epithelial cells derived from human prostate adenocarcinoma and a good system for basic anticancer investigations on prostate cancer [22]. The present study was designed to evaluate the ferutinin contents and cytotoxic effects of roots of *Ferula* species distributing in Anatolia on PC-3 cells.

2. RESULTS

2.1. UPLC analysis

Retention time was 10.58 and equation of the regression line formula and correlation coefficients were Y =-17497.7+119420 and R^2 = 0.9999 for ferutinin (Figure 2). The limit of detection (LOD) based on a signal-to-noise ratio (S/N: 3) was 0.153 µg/ml. The limit of quantitation (LOQ) based on the signal to-noise ratio (S/N: 10) using the lowest concentration in the calibration curve and the highest noise observed when injecting a blank was 510 µg/g. The proposed method was applied to determine ferutinin content in chloroform and hexane extracts of 12 *Ferula* species. Among them ferutinin was detected in *F. tenuissima, F. halophila*, *F. elaeochytris* and *F. duranii* both chloroform and hexane extracts. The highest amount was determined in *F. tenuissima* and *F. halophila* hexane extracts (167380 µg/g and 157160 µg/g respectively). A representative UPLC chromatograms were shown in Figure 1. The content of ferutinin in samples were listed in Table 1.

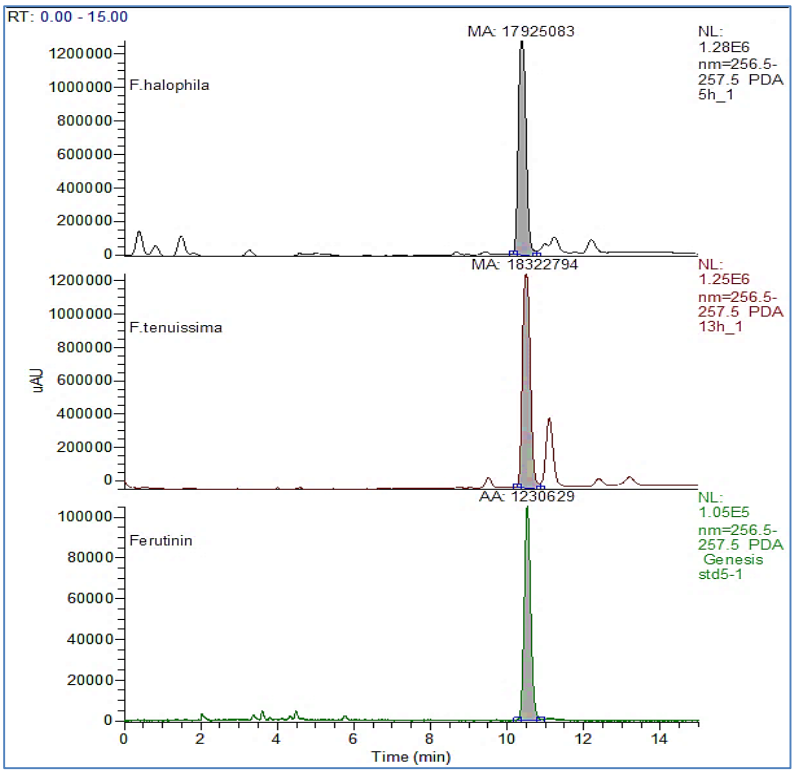

Figure 1. UPLC chromatograms of *F. halophila*, *F. tenuissima* hexane extracts and ferutinin.

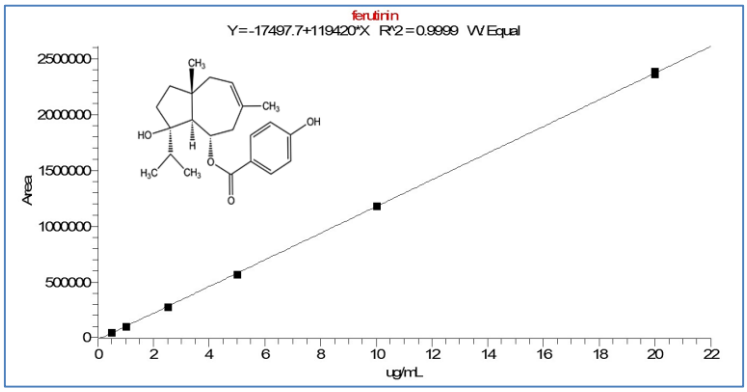

Figure 2. Ferutinin calibration curve (x: Concentration y: Area).

Table 1. Ferutinin contents of *Ferula* L. species.

Species	Amount (ug/g)	
	Chloroform	n-hexane
F. communis* subsp. *communis	NF	17739±0.027
F. drudeana	NF	NF
F. duranii	14380±0.001	32940±0.021
F. elaeochytris	5380±0	31590±0.006
F. halophila	55820±0.02	**157160**±0.258
F. huber-morathii	NF	2241
F. lycia	NF	NF
F. orientalis	NF	19943±0.138
F. rigidula	NF	1321±0.001
F. szowitsiana	NF	779±0.003
F. tenuissima	78660±0.003	**167380**±0.028
F. tingitana	NF	796±0.001

NF: Not found, SD: Mean ± SD (n=3)

2.2. Cytotoxic activity

IC_{50} values of *Ferula* species tested via WST proliferation assay are given in Table 2. Some of the extracts were found to induce cell cytotoxicity with low IC_{50} values. Especially highest activities were observed for *F. elaeochytris* (3.21±0.24 µg/ml) and *F. drudeana* (5.92±0.53 µg/ml) chloroform extracts. *F. szowitsiana* hexane, chloroform extracts (8.03±0.51 µg/ml and 11.14±2.85 respectively) and *F. tenuissima* hexane extract (11.64±1.15 µg/ml) also produced significant inhibition on cells (Figure 3). Methanol fractions of all tested species showed no effect on cell viability. Besides *F. tingitana* chloroform extract showed dose dependent increase in cell proliferation.

Table 2. IC_{50} values of different extracts of *Ferula* L. species.

Species	IC_{50} (µg/ml)		
	n-Hexane	Chloroform	Methanol
F. communis* subsp. *communis	17.94±0.66	40.55±3.89	NA
F. drudeana	14.49±2.36	**5.92±0.53**	NA
F. duranii	61.46±3.23	36.24±14.63	NA
F. elaeochytris	25.55±2.87	**3.21±0.24**	NA
F. halophila	34.7±0.4	38.14±2.53	NA
F. huber-morathii	59.75±1.44	39.82±1.74	NA
F. lycia	35.54±1.12	44.32±1.84	NA
F. orientalis	24.78±0.88	59.31±2.52	NA
F. rigidula	60.72±2.02	58.88±1.79	NA
F. szowitsiana	8.03±0.51	11.14±2.85	NA
F. tenuissima	11.64±1.15	17.64±1.37	NA
F. tingitana*	23.9±0.52	NA	NA

Data are presented as means ± standard deviation (SD).
NA: Not Active (IC_{50}>100 accepted as NA)
* Dose dependent proliferation was determined

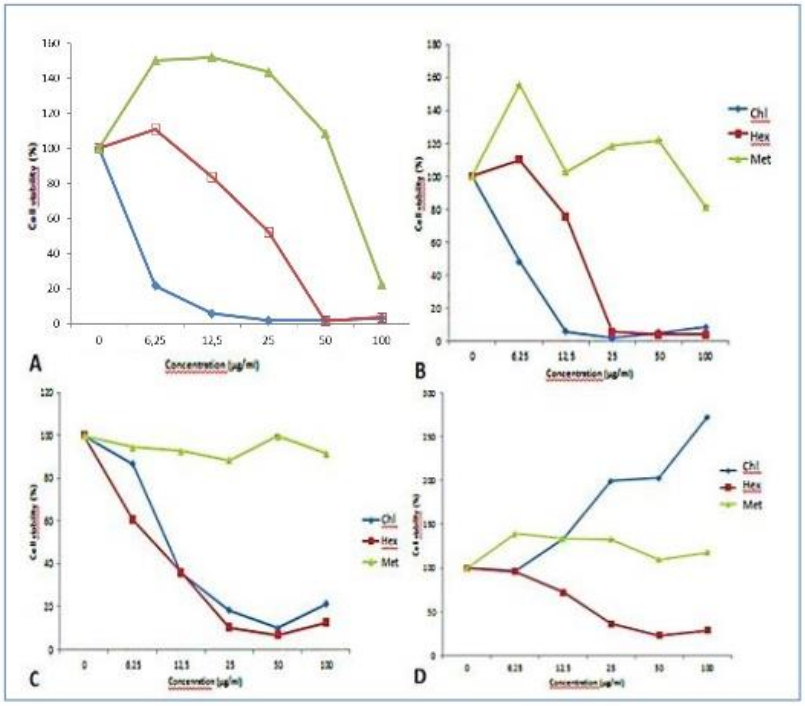

Figure 3. Dose response curves of selected *Ferula* extracts on PC-3 cells **A.** *F. elaeochytris* **B.** *F. drudeana* **C.** *F. szowitsiana* **D.** *F. tingitana (*x: concentration (µg/ml), y: cell viability (%).

3. DISCUSSION

Ferutinin is a daucane type sesquiterpene ester which was first described in 1973 by Saidkhodzhaev A from *F. tenuisecta* [24]. It has been reported from several *Ferula* species such as *F. communis, F. elaeochytris, F. orientalis, F. tenuissima, F. halophila* and *F. rigidula* to date [25–34]. Like phytochemical reports, in our study ferutinin was determined in all these *Ferula* species. Previous studies on *F. szowitsiana* and *F. tingitana* could not lead the isolation of this compound possibly due to the sparingly amount of ferutinin [28,35]. Moreover, to the best of our knowledge this is the first report of this compound found also in *F. duranii* and *F. hubermorathii*.

In our study 12 *Ferula* species were tested for cytotoxic activities and significant results were observed on especially chloroform and hexane extracts. None of the methanol extract had caused cell death on PC-3. Up to now *Ferula* species have been reported to have cytotoxic properties and most of the them were associated with especially nonpolar sesquiterpene contents. So, it can be said that also in our extracts nonpolar molecules are responsible for the cytotoxic activity. The significant result observed by *F. elaeochytris* chloroform extract is in agreement with the previous report of Alktahib et al who were tested sesquiterpene esters isolated from *F. elaeochytris* on leukemia cell lines (K562R (imatinib-resistant) human chronic myeloid leukemia and DA1-3b/M2BCR-ABL (dasatinib-resistant) mouse leukemia cell line) and found the compound elaeochytrin-A to be the most active compound on both cell lines (IC_{50} = 12.4 and 7.8 µM, respectively) [9]. Ferutinin was tested in the same study and showed cytotoxic activity at IC_{50}: 25.3 and 29.1 µM. The double bond between C8/C9 in the molecular structure marked to decrease the activity [9]. Similarly, it is difficult to depend the activity to ferutinin and to mention a parallel correlation between ferutinin amount and the cytotoxicity on PC-3 in our study. *F. elaeochytris* (3.21±0.24 µg/ml) and *F. drudeana* (5.92±0.53 µg/ml) chloroform extracts showed the highest activities but ferutinin content was not observed in same ratio. Nonetheless *F. tenuissima* and *F. halophila* hexane extracts including the highest amount of ferutinin (167380 µg/g and 157160 µg/g respectively) among all extracts didn't provide the best inhibition on the proliferation. Additionally, in another paper published by our group ferutinin isolated from *F. tenuissima* was tested for its cytotoxic activity against PC-3 cell line and IC_{50} value was found as 19.7 µM [23].All these results indicate that ferutinin is not the only responsible of the activity but the total extract including also the other daucane esters causes the effect by synergic activity. And, it seems possible to say that these are nonpolar derivatives since any methanol extract possesses cytotoxic activity.

F. drudeana is an endemic species of Anatolia. Chloroform extract of the plant has a valuable effect on PC-3 proliferation. Dall'Acqua et al reported the antiproliferative effect of daucane sesquiterpenoids against a panel of human tumor cell lines and indicated the importance of structure-activity relationships [35]. Further detailed investigation of sesquiterpenes and sesquiterpene coumarins of *F. drudeana* and its cytotoxic effects is important to discover lead compounds for prostate cancer. *F. tingitana* chloroform extract interestingly caused a high proliferation on the cell line (Figure 3-D). Since the cell proliferation is an important part of tissue repair process, *F. tingitana* found to be a good candidate for wound healing investigations.

4. CONCLUSION

In conclusion, twelve *Ferula* species distributed in Anatolia were investigated for their cytotoxic effects on PC-3 cells and ferutinin contents were evaluated. *F. elaeochytris* and *F. drudeana* showed highest activities and there was not any correlation between ferutinin content and activity. Activity detected in the nonpolar fractions suggests the sesquiterpenes should be the responsible of the activities.

5. MATERIALS AND METHODS

5.1. Plant Materials

The roots of 12 *Ferula* species were collected from different origins of Anatolia in between 2013-2015 at the flowering stages. Plants were identified by the authors and voucher specimens have been deposited in the herbarium of Ege University (IZEF), Faculty of Pharmacy, Izmir, Turkey (www.izef.ege.edu.tr). Details of the collection localities and dates are presented in Table 3.

Table 3. Collection sites, voucher specimens of *Ferula* L. species.

Plant name	Locality	Herbarium no (IZEF)
F. communis subsp. communis	Izmir, Selcuk, Near Ephesus,50 m	5519
F. drudeana*	Kayseri, Yahyalı, 1529 m	5520
F. duranii*	Antalya, Akseki, Çukurköy, 1500 m	5521
F. elaeochytris	Kayseri, Yahyalı, 1200m	5522
F. halophila*	Konya, Cihanbeyli, Tuzgölü, 900m	5523
F. huber-morathii*	Muş-Varto road 40.km, 1280 m	5524
F. lycia*	Antalya, Elmalı, 1030 m	5525
F. orientalis	Muş-Varto road 40.km, 1280 m	5527
F. rigidula	Malatya, Doganshehir, 1280 m	5528
F. szowitsiana	Konya, Cihanbeyli, Tuzgölü, 900m	5529
F. tenuissima*	Osmaniye, Yarpuz, 940 m	6046
F. tingitana	Mugla, Yılanlı Mountain, 860 m	5530

*Endemic species in Turkey

5.2. Extraction

Dried and ground roots (10 g each) of the plants were extracted sequentially with n-hexane, chloroform and methanol at room temperature three times each, in an ultrasonic bath (3 × 300 ml, 3 h for each). The combined extracts were evaporated separately under reduced pressure to dryness at 40 °C using Buchi Rotavapor. Dried extracts were stored at 4 °C until studied. Yields of the extracts are given in Table 4.

5.3. UPLC analysis

5.3.1. Preparation of the standard and samples

Standard ferutinin was isolated and purified in our laboratory from *Ferula tenuissima* and the kindly provided by Aydogan et al. [23]. A stock solution of standard was prepared in chloroform in a concentration of 1 mg/ml. Serial dilutions of the stock solutions were made to obtain 0.5-20 µg/ml. 10 µL of triplicate was

injected into each standard solution to see the reproducibility of the detector response at each concentration level. The peak areas obtained from the injections were plotted against the concentrations to establish the calibration graph. For each plant sample, a solution of 1 mg of dry extract per mL of hexane and chloroform was prepared. All solvents were filtered through a 0.45 µm filter prior to use and degassed in an ultrasonic bath.

Table 4. Extraction yields of *Ferula* L. species.

Species	Extraction Yield (%)		
	n-hexane	Chloroform	Methanol
F. communis subsp. communis	3.04	0.87	4.18
F. drudeana	2.45	4.68	5.86
F. duranii	5.65	0.94	5.69
F. elaeochytris	3.96	1.54	1.22
F. halophila	14.77	3.9	8.69
F. huber-morathii	3.52	1.26	2.23
F. lycia	2.87	2.57	7.49
F. orientalis	5.3	0.78	6.06
F. rigidula	8.88	3.41	8.45
F. szowitsiana	1.53	0.75	5.28
F. tenuissima	0.91	1.96	0.66
F. tingitana	4.95	0.95	6.72

5.3.2. UPLC conditions

The analysis was performed with a system consist of Thermo Scientific Accela (USA, San Jose)–UPLC equipped with solvent degasser, binary pump, auto sampler and PDA detector. Separations were carried out using HICHROM C-18 column (250 mm ×4.6 mm i.d., 5 µm). A flow rate of 1000 µl/min of isocratic water/acetonitrile (30:70 v/v) was performed for 15 min. per sample. The injection volume was 10 µl and detection wavelength for PDA was 257 nm. Quantitative evaluation of standards and samples was performed via peak area using the chromatography software XCalibur (Thermo Fisher Scientific). All analyses were triplicated. Linearity (between 5 equidistant points at the concentrations of 0.5 and 5.0 mg/mL−1, observing the model-fitting through Analysis of Variance – ANOVA, 95%. The limits of detection (LOD) were established at a signal to noise ratio (S/N) of 3. The limits of quantification (LOQ) were established at a signal to noise ratio (S/N) of 10. LOD and LOQ were experimentally verified by the nine injections of reference compounds in LOQ concentrations.

5.4. Cytotoxic activity

5.4.1. Cell Culture

The PC-3 cells were obtained from American Type Culture Collection (ATCC Manassas, VA). PC-3 cells were propagated using DMEM F-12 supplemented with 5% FBS, L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37 °C with 5% CO_2.

5.4.2. WST1 Proliferation Assay

PC-3 ($8x10^3$) cells were seeded and grown in 96-well plates and incubated for 24 hours. Molecule treatments were performed for 48 hours and WST1 cell proliferation reagent (Roche Cat No: 05015944001) was used as recommended. Briefly, WST1 (1:10 final dilution) was added onto the cells at the end of treatments, and the cells were incubated for an additional 3 hours. At the end of the incubation, absorbance measurements at 450 and 690 nm reference wavelengths were performed to calculate the growth rate. Data are presented as means ± standard deviation (SD). GraphPad prism 7.0 and The Student's t test with two-tailed equal variance was applied to assess the statistical significance between pairs when necessary. $P<0.05$ was accepted as significant.

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– S.B., F.A., M.B.Ö., B.D.B., C.Y., B.Ö.; Literature Search – S.B., F.A., M.B.Ö., B.D.B., C.Y., B.Ö.; Writing – S.B., F.A., M.B.Ö., B.D.B., C.Y., B.Ö.; Critical Reviews – S.B., F.A., M.B.Ö., B.D.B., C.Y., B.Ö.

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