Cytotoxic Activity of Sesquiterpenoids Isolated from Endemic Ferula tenuissima Hub.-Mor & Peşmen

Endemik Ferula tenuissima Hub.-Mor & Peşmen'den Elde Edilen Seskiterpenoidlerin Sitotoksik Etkisi

Objectives: This was a phytochemical study of endemic Ferula tenuissima roots and determined the cytotoxic activity of pure compounds on PC-3.

Materials and Methods: Air-dried and powdered roots of F. tenuissima (1 kg) were extracted consecutively with n-hexane, chloroform (CHCl₃), and methanol (MeOH) (3×2 L, each) by sonication at 30°C for 24 h. The extracts were then filtered. The solvents were separately evaporated under reduced pressure to dryness. The compounds were isolated by chromatographic methods and their structures were determined by spectral methods (1D and 2D NMR and LC-MS). The compounds were tested for their cytotoxic activities versus the PC-3 cell line by WST assay.

Results: A phytochemical investigation of the dried roots of endemic F. tenuissima was performed and three sesquiterpene esters were isolated. The daucane-type sesquiterpenes teferidin, ferutinin, and elaechochyrin-A were identified. In the bioactivity study, ferutinin exhibited the highest cytotoxic activity, with an IC₅₀ value of 19.7 µM.

Conclusion: The results indicate that the main compounds of F. tenuissima roots are daucane sesquiterpenes and ferutinin has a potential effect on PC-3 cells.

Key words: Ferula tenuissima, daucane sesquiterpene esters, cytotoxicity, prostate cancer

ÖZ

Amaç: Endemik Ferula tenuissima köklerinin fitokimyasal yönenden incelenmesi ve elde edilen saf bileşiklerin PC-3 üzerinde sitotoksik etkilerinin belirlenmesidir.

Gereç ve Yöntemler: F. tenuissima'nnn açık havada kurutulmuş ve toz haline getirilmiş kökleri (1 kg, 24 saat boyunca 30°Cde sonikasyonda n-hexane, kloroform (CHCl₃) ve metanol (MeOH) (3×2 L, her biri) ile sırasıyla ekstre edilmiştir. Ekstraktlar daha sonra suzilla, sıvı ekstreler, vakum altında kurulguna kadar uçurulmuştur. Kromatografik yöntemler ile bileşikler elde edilmiş ve bileşiklerin yapıları spektral yöntemler ile belirlenmiştir (1D- ve 2D NMR ve LC-MS). Bileşiklerin PC-3 üzerindeki sitotoksik aktiviteleri WST yöntemi ile test edilmiştir.

Bulgular: Endemik Ferula tenuissima'nnun kurutulmuş köklerinin fitokimyasal incelemesi yapılıp ve üç seskiterpen esteri izole edilmiştir. Teferidin, ferutinin ve elaechochyrin-A daucan tip seskiterpen esterleri olarak belirlenmiştir. Biyoaktivite çalışmasında, ferutinin en yüksek sitotoksik aktiviteli IC₅₀: 19.7 µM ile göstermiştir.

Sonuç: Ferula tenuissima köklerinin ana bileşiklerinin daucan seskiterpenler ve ferutininin PC-3 hücreleri üzerinde potansiyel bir etkiye sahip olduğunu göstermektedir.

Anahtar kelimeler: Ferula tenuissima, daucan seskiterpen ester, sitotoksik, prostat kanseri
INTRODUCTION

In recent years, cancer has become the main cause of public health problems and the second leading cause of death in the world despite advanced imaging and molecular diagnostic techniques. Prostate cancer is the most common cancer in men and is the second leading cause of cancer deaths in the United States after lung cancer. Today, most cancer drugs used as cytotoxic agents are obtained directly from natural products like plants, marine organisms, and microorganisms or indirectly by the semisynthesis of molecules from these sources. As a result, cancer research on natural products is expanding.

The genus *Ferula* L. of the family Apiaceae (Umbelliferae) is represented by about 185 species worldwide and 23 taxa in Turkey. Several species, such as the roots of *Ferula gummosa* and *Ferula asafoetida*, have been used in folk medicine as an antidote in poisonings and as an aphrodisiac, antimicrobial, expectorant, and anthemorrhoidal, as well as to treat stomachache, colitis in infants, asthma, and urinary tract disorders. Monoterpenes, sesquiterpenes (especially daucane-, humulane-, and guaiane-type sesquiterpene esters and sesquiterpene lactones), and coumarins were found to be the main constituents of the genus *Ferula* by phytochemical studies. Phenylpropanoid, sulfur-containing derivatives, and triterpenes and their glycosides were also reported. Recent pharmacological research has demonstrated that different extracts of *Ferula* species contain sesquiterpene derivatives that have in particular proven to be cytotoxic on several cancer cell lines. In addition, the extracts have been reported to have antimicrobial, anthelmintic, anticonvulsant, antispasmodic, antihyperglycemic, antihyperlipidemic, and antioxidant activities.

EXPERIMENTAL

Plant material

The roots of *Ferula tenuissima* Hub.-Mor & Peşmen were collected in the Yarpuz Region, Osmaniye, Turkey (940 m) in June 2013. The whole plant was identified by Assoc. Prof. Serdar Gokhan Senol from the Section of Botany, Department of Biology, Faculty of Science, Ege University. A voucher specimen (IZEF 6046) was deposited in the Herbarium of Ege University, Faculty of Pharmacy, Izmir, Turkey (www.izef.ege.edu.tr).

Extraction and isolation

Air-dried and powdered roots of *F. tenuissima* (1 kg) were extracted consecutively with n-hexane, chloroform (CHCl₃), and methanol (MeOH) (3×2 L, each) by sonication at 30°C for 24 h. The extracts were then filtered. The solvents were separately evaporated under reduced pressure to dryness. Yields were 44.31 g, 9.90 g, and 45.89 g, respectively. Next 9.27 g of the CHCl₃ extract was submitted to silica gel column chromatography and eluted consecutively with n-hexane: EtOAc gradient (100:0-0:100, v/v, 10% decreasing polarity) and then acetone: MeOH (100:0-0:100, v/v, 10% decreasing polarity) to give 15 fractions, named A-O, and monitored by thin-layer chromatography (TLC). Based on the TLC profiles 3 fractions, i.e. fractions B (210 mg), E (233 mg), and G (200 mg), were selected for further purification. Fraction B was chromatographed over a silica gel column (150 g) with n-hexane: EtOAc (100:0-87.5:12.5, with 2.5% increasing polarity) to afford five fractions (B1-B5). Fraction B3 (83 mg) was rechromatographed over a silica gel column (75 g) with n-hexane: EtOAc (100:0-90:10, 2% decreasing polarity) to yield compound 1 (40 mg) purely. Then 33 mg of fraction E was further purified by preparative TLC (n-hexane:EtOAc, 80:20, silica gel) and isolated and yielded 17 mg of compound 3. Fraction G was submitted to silica gel column chromatography, eluted with n-hexane: EtOAc (95:5-50:50; 5% increasing polarity) solvent, and yielded compound 2 (33 mg).

Cytotoxicity assay and cells

Cell toxicity was analyzed by using WST-1 according to the manufacturer’s protocol. PC-3 and RWPE-1 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). PC3 cells were propagated using DMEM F-12 supplemented with 5% FBS, L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL), while RWPE-1 cells were propagated in keratinocyte growth medium supplemented with bovine pituitary extract and 5 µM EGF at 37°C with 5% CO₂. Molecules were dissolved in DMSO and treatments were done so that DMSO volume would not exceed 0.5% of the culture media volume. Control cells were treated with the same volume of DMSO used during the molecule treatments. PC-3 (100 103) and RWPE-1 cells (104) were seeded and grown in 96-well plates and incubated for 24 h. Molecule treatments were performed for 48 h and WST-1 cell proliferation reagent (roche cat no: 05015944001) was used as recommended. Briefly, WST-1 (1:10 final dilution) was added to the cells at the end of treatments, and the cells were incubated for an additional 3 h. At the end of the incubation, absorbance was measured at 450 and 690 nm using a SpectraMaxPlus 384 spectrophotometer (Molecular Devices). IC₅₀ concentrations of the molecules were calculated through nonlinear regression analysis in GraphPad Prism 6. All experiments were performed in triplicate. Doxorubicin was used as the positive control.

Chemicals and other materials

Mass spectra (Thermo-Scientific TSQ Quantum Access Max LC-MS/MS, ESI+). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Oxford AS400 and a Bruker DRX-500. The chemical shifts were measured relative to the residual solvent peak and are expressed in δ (ppm) and the coupling constants (J) are reported in Hertz (Hz). Column chromatography was carried out on silica gel 60 (40-63 mm, Merck), Sephadex LH-20 (GE Healthcare), and Micropore RP-18 (25-40 mm, Merck) using analytical grade purity solvents (Merck and Sigma). TLC analyses were carried out on silica gel 60 F254 and RP-18 F254s (Merck) precoated aluminum plates. Compounds were detected by UV (244-366 nm) and 10% vanillin ethanol solution/H₂SO₄ reagent followed by heating at 105°C for 1-2 min.
RESULTS AND DISCUSSION

The powdered roots of *F. tenuissima* were extracted consecutively with n-hexane, CHCl₃, and MeOH (3×2 L, each) by sonication at 30°C for 24 h. The CHCl₃-soluble fraction was subjected to repeated column chromatography (CC) to afford three known compounds (see Figure 1). All of them were daucane-type sesquiterpenoids; their structures were established by NMR and MS and by comparison with published data. Compounds 1 (teferidin), 2 (ferutinin), and 3 (elaeochytrin-A) were also identified by comparison of their spectral data with those in the literature.

**Compound 1 (Teferidin)**

4β-Hydroxy-6α-benzoxyloxy-5α(H)-dauc-8-ene: Yellow residue, EI MS/MS [M+H]⁺ at *m/z* 359.06, [M+NH₄]⁺ *m/z* 376.11, [M+Na]⁺ *m/z* 381.06, [M+K]⁺ *m/z* 397.03 for C₂₂H₃₀O₅; ¹H, ¹³C NMR spectroscopic data, see Table 1.

**Compound 2 (Ferutinin)**

4β-Hydroxy-6α-(p-hydroxy benzoyloxy)-5α(H)-dauc-8-ene: Yellow residue, EI MS/MS [M+H]⁺ at *m/z* 359.06, [M+NH₄]⁺ *m/z* 376.11, [M+Na]⁺ *m/z* 381.06, [M+K]⁺ *m/z* 397.03 for C₂₂H₃₀O₅; ¹H, ¹³C NMR spectroscopic data, see Table 1.

**Compound 3 (Elaeochytrin-A)**

4β-Hydroxy-6α-(p-amino benzyloxy)-5α(H)-dauc-8-ene: Yellow residue, EI MS/MS [M+Na]⁺ *m/z* 380.34 for C₂₂H₃₁NO₃; ¹H, ¹³C NMR spectroscopic data, see Table 1.

All compounds isolated from *F. tenuissima* were evaluated for their cytotoxic activity against the PC-3 cancer cell and normal prostate RWPE cell lines. The IC₅₀ values of compounds that are active on at least one cell line at concentration are given in Table 2.

![Figure 1. Structure of compounds 1-3](image)

| Compound  | R₁  | R₂  |
|-----------|-----|-----|
| 1-Tefedin | H   | H   |
| 2-Ferutinin | O   | H   |
| 3-Elaeochytrin-A | H   | NH₂ |

CONCLUSION

Mono-, di-, triesters of humulane, germacrane, eudesmane, and especially daucane-type sesquiterpenes and coumarin and lactone derivates are major components of the genus *Ferula* L. It was observed that the location of the double bond in the daucane ring affected activity, which was at positions 7-8, 8-9, and 9-10. Furthermore, the presence of hydroxyl groups at different positions on the daucane ring and the formation of mono-, di-, and tri-ester structures of this hydroxyl group, especially benzoic, angelic, cinnamic, and vanillic acid, increases the variability in biological activity. The isolated teferidin compound is jaeschkeanadiol benzoic acid ester isolated from *F. tenuisecta* roots for the first time in 1976. It has also been reported from *F. hermonis*, *F. pallida*, *F. elaeochytris*, *F. rigidula*, and *F. jaeschkeana* roots. Ferutinin was first described in 1973 by Saidkozev as jaeschkeanadiol p-hydroxy benzoic ester. It was isolated from different *Ferula* species previously. Elaeochytrin-A was first reported from *F. elaeochytris* roots.

In our study, it was determined that all compounds were moderately effective on the PC-3 and RWPE-1 cell lines. The affinity of the compounds for RWPE-1 cells also indicated that the selectivity of the compound is not as high as expected.

In a previous study, elaeochytrin-A showed cytotoxic effects on K562R (imatinib-resistant) human chronic myeloid leukemia and DAI-3b/M2BCR-ABL (dasatinib-resistant) mouse leukemia cell lines at IC₅₀ 12.4 and 7.8 µM concentrations, respectively. In the same study, ferutinin showed cytotoxic activity at IC₅₀ 25.3 and 29.1 µM and teferidin at IC₅₀ 55.1 and 29.5 µM. When the molecular structures were examined, the double bond between C8 and C9 positions decreased cytotoxic activity. Ferutinin has been shown to have an antiproliferative effect on colon cancer cell lines of WiDr, COLO320-HSR, and LS-174T and to induce apoptosis and intracellular Ca²⁺ pathway in human Jurkat cells. Ferutinin showed ERα and ERβ agonist and antagonist receptor activity with improving sexual function in male and female rats. It has been found that the hydroxyl group at position 3 increases the estrogen-like effect of the presence of oxygen and the presence of electrophilic groups in the p-position of the benzene ring (hydroxyl, o xo, etc.). Prostate cancer formation, especially androgenic hormones, is the main cause of uncontrolled proliferation of cells. Ferutinin molecule studies suggest that both the effects on sexual function and the activity of *in vitro* cytotoxicity studies may be specific antagonist/agonist effects on androgen hormone receptors. As a result of our bioactivity studies on the PC-3 cell line with compounds isolated from *F. tenuissima* roots, the most active cytotoxic agent of ferutinin synthesis emerged (IC₅₀ 19.69 µM), in addition to the potential phototherapeutic results of phytochemical and bioactivity studies of other genus-related species due to the biological activity of the root extracts of *Ferula* taxa and daucane-type sesquiterpenoids.

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Table 1. NMR spectroscopic data for compounds 1-3 in CDCl₃ (δₗ 7.26 ppm, δₛ 77.1 ppm)

| Positions | Compound 1 | Compound 2 | Compound 3 |
|-----------|------------|------------|------------|
| δₗ (δ ppm, J=Hz) | δₛ | δₗ (δ ppm, J=Hz) | δₛ | δₗ (δ ppm, J=Hz) | δₛ |
| 1         | -          | 43.9 (s)   | -          | 44.0 (s)   | -          | 44.2 (s) |
| 2         | 1.43 (m)   | 1.55 (m)   | 1.54 (m)   | 41.5 (t)   |
| 3         | 1.92 (m)   | 1.92 (m)   | 1.91 (m)   | 31.8 (t)   |
| 4         | -          | 85.1 (s)   | -          | 87.0 (s)   | -          | 86.5 (s) |
| 5         | 1.96 (d, J=9.6) | 59.4 (d) | 2.02 (d, J=10.8) | 60.1 (d) | 2.00 (d, J=10.8) | 60.1 (d) |
| 6         | 5.09 (td, J=10.2, 2.4) | 70.9 (d) | 5.27 (td, J=10.4, 2.8) | 71.2 (d) | 5.27 (ddd, J=10.8, 10.4, 2.8) | 70.7 (d) |
| 7         | 2.39 (dd, J=12.0, 10.8) | 41.5 (t) | 2.25 (dd, J=12.4, 11.2) | 2.27 (dd, J=14.0, 2.4) | 41.6 (t) |
| 8         | -          | 133.7 (s) | -          | 133.5 (s) | -          | 134.4 (s) |
| 9         | 5.50 (bs)  | 125.3 (d) | 5.55 (bt, J=5.6) | 125.3 (d) | 5.55 (bs) | 125.4 (d) |
| 10        | 1.96 (m)   | 40.9 (t)  | 2.06 (m)   | 41.0 (t)  | 2.05 (m)  | 41.2 (t)  |
| 11        | 2.10 (sept, J=6.8) | 36.5 (d) | 1.86 (sept, J=6.8) | 37.0 (d) | 2.04 (m) | 37.4 (d) |
| 12        | 0.75 (d, J=6.8) | 18.1 (q) | 0.85 (d, J=6.8) | 17.6 (q) | 0.85 (d, J=6.8) | 17.7 (q) |
| 13        | 0.94 (d, J=6.8) | 18.8 (q) | 0.94 (d, J=6.8) | 18.5 (q) | 0.95 (d, J=6.8) | 18.7 (q) |
| 14        | 1.01 (s)   | 20.6 (q)  | 1.10 (s)   | 20.2 (q)  | 1.11 (s)  | 20.3 (q)  |
| 15        | 1.75 (s)   | 26.6 (q)  | 1.81 (s)   | 26.4 (q)  | 1.82(s)   | 26.6 (q)  |
| 1₁        | -          | 131.3 (s) | -          | 121.9 (s) | -          | 111.0 (s) |
| 2₁        | 7.89 (d, J=7.2) | 129.3 (d) | 7.92 (d, J=8.8) | 132.0 (d) | -          | 151.1 (s) |
| 3₁        | 7.50 (dd, J=8.0, 7.2) | 129.0 (d) | 6.88 (d, J=8.8) | 115.5 (d) | 6.67 (dd, J=8.0, 0.8) | 117.0 (d) |
| 4₁        | 7.61 (t, J=7.2) | 133.3 (d) | -          | 161.1 (s) | 7.27 (ddd, J=7.2, 6.8, 1.6) | 133.7 (d) |
| 5₁        | 7.50 (dd, J=8.0, 7.2) | 129.0 (d) | 6.88 (d, J=8.8) | 115.5 (d) | 6.64 (td, J=8.0, 1.2) | 116.4 (d) |
| 6₁        | 7.89 (d, J=7.2) | 129.3 (d) | 7.92 (d, J=8.8) | 132.0 (d) | 7.78 (dd, J=8.0, 1.6) | 131.0 (d) |
| 7₁        | -          | 165.2 (s) | -          | 167.3 (s) | -          | 168.3 (s) |

F. tenuissima showed a profile of chloroform extracts with ultraviolet active at 254 and 366 nm, blue-green color visible spots with vanillin/H₂SO₄ reagent for all compounds. NMR: Nuclear magnetic resonance.

Table 2. Cytotoxicity (IC₅₀ in μM) of isolated jaeschkeanadiol esters against prostate cancer cell lines in vitro

|          | PC-3      | RWPE-1    |
|----------|-----------|-----------|
| Teferidin| 65.3±4.10 | 21.77±1.20|
| Ferutinin| 19.69±2.22 | 3.295±0.80 |
| Elaeochytrin-A | 44.23±3.27 | 8.299±0.9 |
| Doxorubicin | 1.17±0.12 | 0.468±0.038 |

* Data are mean values ± standard deviation of three experiments, IC₅₀ μM, positive control, PC-3 (Prostate cancer cell line), RWPE-1 (Normal prostate epithelial cell line).

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