RESEARCH ARTICLE

Ferutinin, an Apoptosis Inducing Terpenoid from *Ferula ovina*

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Abstract

A current hurdle in cancer management is the intrinsic or acquired resistance of cancer cells to chemical agents that restricts the efficacy of therapeutic strategies. Accordingly, there is an increasing desire to discover new natural compounds with selective toxicity to combat malignancies. In present study, the cytotoxic and apoptosis-inducing activities of ferutinin, a terpenoid derivative from *Ferula ovina*, were investigated on human breast (MCF7) and bladder (TCC) cancer cells as well as normal fibroblasts (HFF3).The toxicity and DNA damage inducing effects of ferutinin were studied by MTT and comet assays, DAPI and PI staining and DNA laddering. The IC_{50} values of ferutinin were identified and compared with routine prescribed drugs, doxorubicin and vincristine, by MTT test. Alkaline comet assay and DAPI staining revealed DNA damage due to ferutinin, which was significantly ($p<0.001$) higher in MCF7 and TCC than HFF3 cells. Apoptosis induction was evidenced by PI staining and DNA laddering. Our results suggest that ferutinin could be considered as an effective anticancer agent for future *in vivo* and clinical experiments.

Keywords: Anticancer - ferutinin - apoptosis - *Ferula ovina*

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Introduction

Cancer is the second leading cause of death in the world. In 2005, the global incidence of cancer was 11 million and it is expected to increase to an incidence of 15.5 million by 2030 (Strong et al., 2008). Breast carcinoma, the most commonly occurring cancer in women worldwide, and transitional cell carcinoma (TCC) of the bladder, the most prevalent malignancy of the urinary system, are two types of cancers which have shown increasing statistics in recent years (Ferlay et al., 2010). Although a lot of progress has been achieved in cancer therapy and management, the existence of resistant cancer cells has restricted the efficiency of current radio and chemotherapy regimens (Rivera and Gomez, 2010). This drawback points to the need of expanding the exploration for novel, more effective and preferably natural compounds with anticancer properties.

Terpenes are a vast group of biosynthetically compounds with diverse pharmacological properties including antifungi (Barrero et al., 2000; Al-Mughrabi and Aburjai, 2003; Saddiq and Khayyat, 2010), antimicrobial (Do Socorro et al., 2003; Chiang et al., 2005; Saddiq and Khayyat, 2010; Kanokmedhakul et al., 2012), anti-inflammatory (Koch et al., 2001; Peana et al., 2002), and antitumor effects (Jagetia, et al., 2005; Chen et al., 2007; Choi and Lee, 2009; Mansoor et al., 2009; Kanokmedhakul et al., 2012; Kolli et al., 2012; Shang-Gao et al., 2012). Genus *Ferula* (Apiaceae) contains more than 130 species, among which 30 species have been represented in Iranian flora (Mozaffarian, 1996). Plants belong to this genus are documented in the traditional medicine of the Middle East area and have been particularly used as potential antiflatulent, antispasmodic and anticonvulsant herbs in Iran (Zargari, 1988). *Ferula* species are good sources of biologically active compounds such as terpenoid coumarins and sesquiterpene derivatives (Iranshahi et al., 2007; Iranshahi et al., 2009) and our previous studies demonstrated that these derivatives could reverse the drug resistance of cancerous cells (Rassouli et al., 2009; Mollazadeh et al., 2010; Rassouli et al., 2011a), and show cytotoxic (Rassouli et al., 2011b), antileishmanial (Iranshahi et al., 2007), and cancer chemopreventive (Iranshahi et al., 2008; Iranshahi et al., 2010) activities.

In present study, ferutinin was isolated from the roots of *F. ovina* and evaluated for its cytotoxic and apoptosis-inducing effects *in vitro*. The investigations were carried out on human breast (MCF7) and bladder (TCC) cancerous cells, and also human foreskin fibroblasts (HFF3). To study the activity of ferutinin, MTT assay, alkaline single cell gel electrophoresis approach (comet assay), 4', 6-diamidino-2-phenylindole (DAPI) and propodium iodide (PI) stainings and DNA laddering were used. Furthermore, to better compare the toxic effects of ferutinin, vincristine

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and doxorubicin, routine anticancer drugs prescribed for bladder and breast malignancies (Gurib-Fakim, 2006; Ibrahim et al., 2000), were also used as positive controls.

Materials and Methods

Extraction and isolation of ferutinin

F. ovina was collected from Binaloud Mountain, north of Iran, in May 2007, and voucher specimens were deposited in the herbarium of the School of Pharmacy, Mashhad University of Medical Sciences under accession No. 1011. The concentrated dichloromethane extract of *F. ovina* roots was subjected to normal phase column (60×5cm) chromatography; the elution of column was done by petroleum ether and subsequently ethyl acetate. The fractions were compared by TLC (Silica gel using petroleum ether-acetone as solvent), and those giving similar spots were combined. Three fractions were finally obtained; fraction 3 was assigned as ferutinin (a sesquiterpene) and its structure was confirmed by 1D- and 2D- nuclear magnetic resonance (NMR) spectra (Figure 1), which was in agreement with those previously described in the literature (Abd El-Razek et al., 2003).

Preparation of reagents' concentrations

Different concentrations of ferutinin (5, 10, 20, 30, 40, 50 and 100 µg/ml) were prepared by dissolving 2 mg of the powder in 100 µl dimethylsulfoxide (DMSO, Merck, Germany) and diluted with complete culture medium before experiments. To exclude the background cytotoxic effects of the solvent, control cells were treated with equivalent amounts of DMSO content in each concentration; 0.025%, 0.05%, 0.1%, 0.15%, 0.2%, 0.25% and 0.5% DMSO.

To better evaluate ferutinin toxicity, different concentrations of vincristine (Richter, Hungary; 25, 50, 100 and 250 µg/ml) and doxorubicin (Ebewe, Austria; 8, 16, 32, 48 and 64 µg/ml) were prepared using complete culture medium right before experiments.

Culture of cancerous and normal cells

MCF7 and TCC cells were purchased from Pasteur Institute (Tehran, Iran), while HFF3 cells were kindly provided by Royan Institute (Tehran, Iran). All cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Scotland) supplemented with 10% fetal bovine serum (Gibco, Scotland) and incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air. To subculture the cells, 0.25% trypsin and 1 mM EDTA were used for 3-5 min.

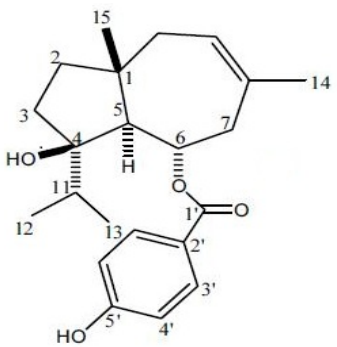

Figure 1. The Chemical Structure of Ferutinin Isolated from *F ovina*

For morphological alterations, treated MCF7, TCC and HFF3 cells with various concentrations of ferutinin were observed under invert microscope 24, 48 and 72h after treatments.

MTT cytotoxicity assay

To determine the half maximal inhibitory concentration (IC_{50}) of all reagents used in present study, MTT assay was used as previously described (Rassouli et al., 2011a). Briefly, 24h after cells were seeded at a density of 8×10^3 cells/well in 96-well plates, they were treated with increasing concentrations of ferutinin, vincristine or doxorubicin for 24, 48 and 72h. Then, 5 mg/ml MTT solution (Sigma, Germany) was added to each well, plates were incubated at 37°C for 4h, and finally insoluble produced formazan was dissolved in DMSO. The optic density of each well was measured spectrophotometrically at 570 nm; all tests were performed in triplicate. The viability of cells in each treatment was calculated by dividing the absorbance of treated cells in each concentration to the mean absorbance of control cells.

Alkaline comet assay and DAPI staining

To semi-quantitatively study the DNA damaging effect of ferutinin, alkaline comet assay and DAPI staining were performed as previously described (Rassouli et al., 2011a). Briefly, untreated MCF7, TCC and HFF3 cells, and cells treated with IC_{50} values of ferutinin, as well as their DMSO controls, were trypsinized and washed in phosphate buffered saline (PBS).

For comet assay, cell pellets were suspended in 0.75% (w/v) low melting point agarose (LMA, Fermentas, Germany), dispensed onto slides precoated with 1% (w/v) normal melting agarose (NMA, Helicon, Russia), solidified on ice, and covered with 0.75% (w/v) LMA. After immersing slides in lysing buffer (pH 10) and 4h incubation at 4°C, they were kept in alkaline electrophoresis buffer (pH 13) for 30 min. Then, electrophoresis was conducted for 20 min at 4°C, in an electric field of 300 mA and 25 V. Afterwards, slides were washed with neutralizing buffer (pH 7.5), dried and stained with ethidium bromide (EtBr), and finally analyzed using a fluorescent microscope (Olympus, Japan). The percentage of DNA in tail, an indicator for DNA fragmentation, was determined using TriTek Cometscore version 1.5 software. For DAPI staining, cell pellets were fixed in 4% paraformaldehyde (Sigma, Germany), permeabilized with 0.1% Triton X-100 and stained with 2 µg/ml DAPI (Merck, Germany) for 10 min. Stained cells were then counted under fluorescent microscope, while chromatin condensation and nuclear fragmentation were the criteria used to demonstrate apoptosis.

Apoptosis assay by PI staining

To determine cancerous cells arrested in sub-G1 phase of the cell cycle, which are known as apoptotic cells, untreated MCF7 cells, cells treated with 37 and 74 µg/ml

ferutinin and their relevant DMSO controls, and also cells treated with 64 µg/ml doxorubicin were suspended in a hypotonic buffer (50 μg/ml PI in 0.1% sodium citrate and 0.1% Triton X-100) and incubated overnight at 4°C. Then, flow cytometric analysis was done using FACS Callibur (BD Biosciences).

DNA fragmentation analysis

To assess oligonucleosomal fragmentation, genomic DNAs of untreated MCF7 cells, cells treated with 0.36% and 0.72% DMSO and cells treated with 74 and 148 µg/ml ferutinin, were extracted by a lysing solution (20 mM Tris-HCl, 20 mM EDTA, 200 mM NaCl and 1% SDS, pH 7.5) containing 2 µl of RNase A (2 mg/ml, Sigma) and proteinase K (CinnaGen, Iran). DNA samples were then loaded onto a 2% agarose gel, electrophoresed at 50 V for 3h and finally stained by EtBr.

Statistical analysis

Significant level was ascertained by one way ANOVA, followed by Tukey multiple comparison test, using SPSS software. Values are expressed as mean±SD. A p-value of <0.001 was considered as significant.

Results

To investigate the cytotoxicity of ferutinin by MTT test, MCF7, TCC and HFF3 cells were treated with increasing concentrations of this sesquiterpene, as well as equivalent DMSO dilutions as controls, and also vincristine and doxorubicin, for 24, 48 and 72h. As summarized in Table 1, ferutinin induced its toxic effects in a dose and time dependent manner; 72h after treatment, the IC_{50} values of ferutinin on MCF7, TCC and HFF3 cells were 29, 24 and 36 μg/ml, respectively. Interestingly, in comparison with this terpenoid, vincristine and doxorubicin induced their toxic effects in higher concentrations, as the IC_{50} values of doxorubicin and vincristine were 64 μg/ml on MCF7 cells and 50 μg/ml on TCC cells, respectively.

Examining morphological alterations revealed that in comparison with untreated and control cultures, treatment with IC_{50} value of ferutinin reduced the number of cells and their attachment, and also induced cytoplasmic granulation in all cell lines (Figure 2).

To determine the mechanisms involved in ferutinin activity, DNA damage was analyzed by alkaline version of comet assay. Based on MTT results and morphological alterations, comet assay was carried out on untreated MCF7 cells, cells incubated with DMSO control, and cells treated with IC_{50} values of ferutinin for 24h (Figure 3A-C). As it is shown, 37 μg/ml ferutinin induced approximately 42% DNA damage, significantly ($p<0.001$) higher than that induced by its relevant controls (Figure 3G).

Due to its great activity, ferutinin effects on nuclear morphology was investigated by DAPI staining on MCF7 cells (Figure 3D-F). Demonstrating apoptotic morphology revealed that 71% of MCF7 cells treated with 37 µg/ml ferutinin presented condensed chromatins and fragmented nuclei, significantly ($p<0.001$) higher than its relevant controls (Figure 3G).

As it is demonstrated in Figure 4A-F, flow cytometry analysis after PI staining revealed that in comparison with untreated MCF7 cells and cells treated with 0.18% and 0.36% DMSO, about 10% and 45% of cells treated with 37 and 74 µg/ml ferutinin were detected in sub-G1 peak, respectively, which is comparable to doxorubicin effects (60%).

To further analyze ferutinin effects on DNA structure, DNA laddering was applied on MCF7 cells treated with 74 and 148 µg/ml ferutinin and their relevant DMSO and untreated controls. As it is presented in Figure 4G, only in cells treated with 74 and 148 µg/ml ferutinin severe DNA fragmentations were observed.

Table 1. IC_{50} Values of Ferutinin, Vincristine and Doxorubicin on Cancerous and Normal Cells. MTT Assay was Done During 3 Consecutive Days

IC_{50} values (μg/ml)	Time duration/ Cell line	24h	48h	72h
Ferutinin	MCF7	37	32	29
	TCC	33	27	24
	HFF3	46	37	36
Doxorubicin	MCF7	>64	>64	64
Vincristine	TCC	70	50	50

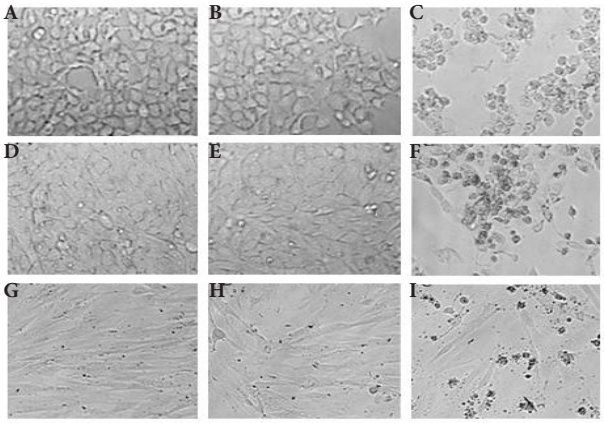

Figure 2. Photomicrographs of MCF7 (A-C), TCC (D-F) and HFF3 (G-I) Cells without any Treatment (A, D and G), Treated with 0.18% (B), 0.16% (E) and 0.23% (H) DMSO (Ferutinin Controls) and Treated with 37 μg/ml (C), 33 μg/ml (F) and 46 μg/ml (I) Ferutinin for 24 h

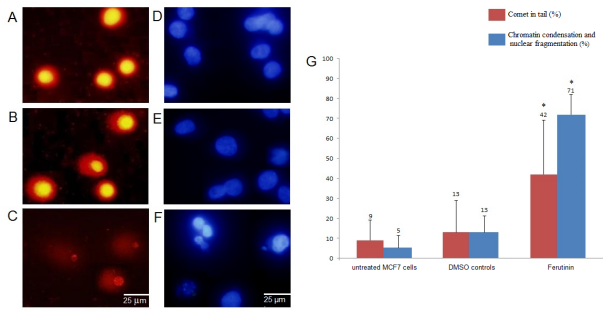

Figure 3. Photomicrographs of DNA Lesions Induced by Ferutinin and its Relevant Controls. MCF7 cells without any treatment (A), treated with 0.18% DMSO (B), and treated with 37 μg/ml Ferutinin (C) for 24 h. The percentages of damaged DNA 24h after treatments (D). Data are expressed as mean±SD; *indicates significant ($p<0.001$) difference between cells treated with ferutinin and other cultures

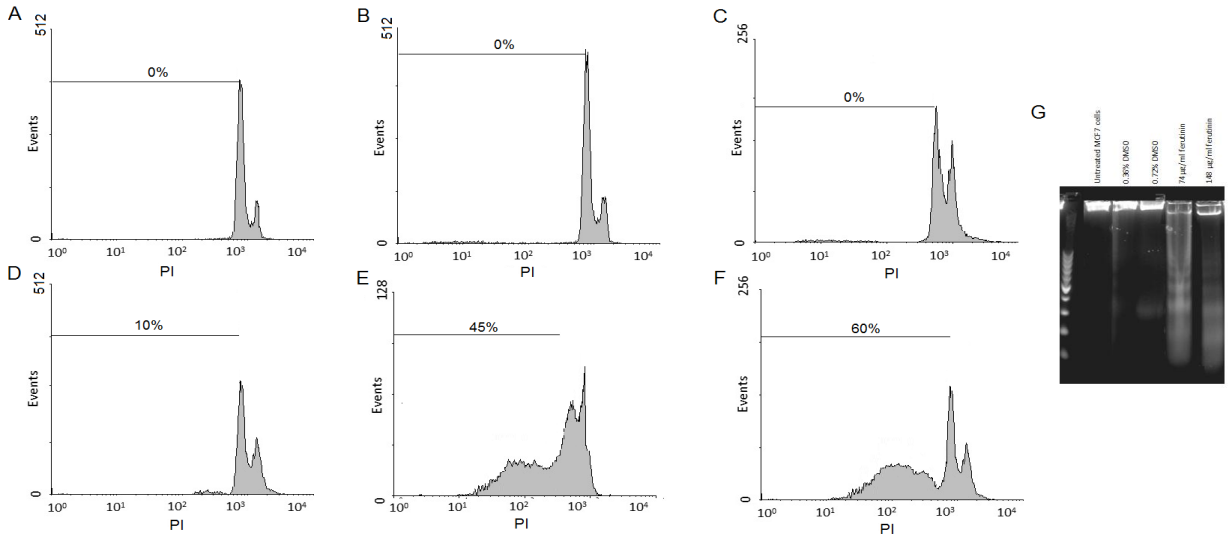

Figure 4. Apoptosis Assay by PI Staining Untreated MCF7 Cells (A), Cells Treated with 0.18% (B) and 0.36% (C) DMSO, Cells Treated with 37 µg/ml (D) and 74 µg/ml (E) Ferutinin, and 64 µg/ml Doxorubicin (F) for 24h. Sub-G1 peak, as an indicative of apoptotic cells, was induced in ferutinin treated cells but not in their controls

Discussion

The intrinsic or acquired resistance of cancer cells to chemical reagents and ionizing radiations is the most important factor that has been introduced for the low efficacy of current therapeutic strategies against malignancies including breast and bladder cancers. This drawback has prompted a good deal of investigations in search for new biosynthetical compounds with antitumor properties.

For years, plant-derived natural products have been used for the development of cancer therapeutic agents in modern medicine. There are several ongoing clinical trials with natural compounds, including green tea, genistein and n-3 poly unsaturated fatty acids that show promising results in the prevention and/or therapy of breast and bladder cancers (Amin et al., 2009). Interestingly, dietary terpenoids have shown antitumor activity on a wide variety of experimental tumors by preventing the formation of neoplastic cancers or regressing the existence of malignant tumors (Crowell et al., 1996; Bardon et al., 1998; Shi et al., 2002; Jagetia et al., 2005).

Ferula species are reputed in traditional medicine for their therapeutic applications against a range of disorders, and terpenoid compounds are the most abundant constituents of *Ferula* essential oils. A number of medicinal properties including antibacterial, antifungal, antioxidant, anti-inflammatory, anticonvulsant and hypotensive activities have been reported for *Ferula* species in several studies (Sahebkar and Iranshahi, 2011). Due to these great therapeutic potentials, we selected ferutinin, extracted from *F. ovina*, to study its probable antitumor and apoptosis inducing activities.

Present results revealed that the IC_{50} value of ferutinin on MCF7 cells were less than those of doxorubicine, a currently prescribed drug for breast carcinoma. Moreover, the toxicity of this terpenoid on TCC cells was also higher in comparison with vincristine, a routine anticancer drug used for bladder cancer treatment. Studying the activity of ferutinin during 72h revealed that this compound act in a dose and time dependent manner and show less toxic effects on normal human fibroblasts.

Apoptosis is a highly complex process that is involved in normal cell turnover, and its misregulation may account for tumor development. Nuclear morphological changes in the form of fragmentation and condensation, and also DNA lesions detected as laddering patterns are known as common and important characteristics of apoptosis. Using alkaline version of comet assay revealed that in comparison with control cultures, ferutinin significantly increased DNA lesions in treated cells. Furthermore, we detected a significant higher number of condensed chromatins in cells treated with ferutinin by DAPI staining, which was in agreement with MTT results and morphological observations, and was also confirmed by PI staining and DNA laddering results.

For many years, ferutinin, an aromatic ester of a daucane alcohol, was known as a potent phytoestrogen that has agonist activities over the α subunit of human estrogen receptor (ER), while acts as agonist/antagonist for ERβ (Ignatkov et al., 1990; Ikeda et al., 2002). Recent studies indicated that ferutinin has acetylcholinesterase inhibitory activity and shows anti-inflammatory and antimicrobial effects (Dall'Acqua et al., 2010; Geroushi et al., 2011; Abourashed et al., 2011). This sesquiterpene promotes the redistribution of intracellular Ca^{2+}-pools throughout different cellular compartments including the release of Ca^{2+} from endoplasmic reticulum into the cytosol and also the accumulation of Ca^{2+} in the mitochondria (Zamaraeva et al., 1997; Abramov et al., 2001; Abramov and Duchen, 2003). Furthermore, ferutinin exhibits antiproliferative effects on breast cancer cells (Lhuillier et al., 2005) and induces apoptosis in leukemia cells through a loss in mitochondrial transmembrane potential and an increase in intracellular reactive oxygen species (Macho et al., 2004). Interestingly, it has been shown that ferutinin increases apoptosis in uterine luminal and glandular epithelia, and as a result, has protective function against uterine carcinoma

(Ferretti et al., 2012). The Ca^{2+}-ionophoretic properties of ferutinin appear to explain its reported toxic effects, since calcium elevation in prelethal concentrations induces mitochondrial permeability, which is closely linked to the induction of apoptosis (Crompton, 1999).

It has been shown that terpenoid derivatives with antitumor effects act through diverse mechanisms including mitochondrial permeability and depolarization (Chen et al., 2007), activation of enzymes such as c-Jun N-terminal kinase, Fas, caspase-8 and caspase-3 (Choi and Lee, 2009; Mansoor et al., 2009; Choi et al., 2012), microtubule polymerization and lipid peroxidation (Bocca et al., 2004; Jagetia et al., 2005), to clarify ferutinin exact mechanism of action, further and precise studies are needed.

In conclusion, *F ovina* is an edible plant that has been widely used in Iranian traditional medicine for its therapeutic effects. Present results indicate that ferutinin, sesquiterpene from the roots of *F. ovina*, has selective cytotoxic effects, and induce apoptosis *in vitro*. Since the binding of ferutinin to albumin protein guarantees the high stability and well distribution of this compound in plasma (Greige-Gergesa et al., 2008), ferutinin could be considered as a suitable antitumor agent for further preclinical studies.

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