Investigating the cytotoxic and apoptosis inducing effects of monoterpenoid stylosin in vitro

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**A R T I C L E   I N F O**

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**A B S T R A C T**

The aim of this study was to investigate the cytotoxic and anticancer activities of stylosin, a monoterpene extracted from an edible plant, *Ferula ovina*, on 5637 and HFF3 cells using MTT and comet assays and DAPI staining.

To assess stylosin effects, cells were cultured in the presence of various concentrations of stylosin during three days; the IC50 of stylosin on cancerous 5637 cells was less than its value on HFF3 normal cells, indicating that it might have anticancer properties. Investigating the mechanism of stylosin action revealed that it quickly induced DNA lesions and increased the number of apoptotic cells.

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1. Introduction

Transitional cell carcinoma (TCC) is a superficial tumor arising from the transitional epithelium lining of the urinary bladder. TCC accounts for approximately 90% of all bladder cancers with more incidence in men [1]. The combination of vinblastine, methotrexate, doxorubicin and cisplatin is the best-studied chemotherapy regimen for TCCs [2]. However, due to resistance of TCC cells to a wide range of chemotherapeutic agents, complete responses have been obtained only in a small proportion of patients [3].

Monoterpenes are naturally occurring hydrocarbons, which have shown antitumour activities on a wide variety of experimental tumours [4–9] and different human cancerous cell lines [7,9–14].

The genus *Ferula* (Apiaceae) has a wide distribution throughout Mediterranean and Middle East area, especially in countries such as Iran. *Ferula* species are well documented as a source of biologically active compounds that are found in different parts of the plants, and due to their diverse and effective pharmaceutical properties, they are widely used as edible plants in several Asian countries. For instance, since *Ferula* species possess stimulant, expectorant and vulnerary effects, they have been traditionally used for the treatment of a number of respiratory and digestive system disorders including asthma, influenza, stomachache, flatulence and intestinal parasites [15–17]. Furthermore, in Iranian traditional medicine, the fresh leaves and yellow concrete gummy-resinous juice of the stems, which is called sagapenum, are edible parts of *Ferula* species that have been widely used for their antiepileptic, antiflatulent, antispasmodic and also anticonvulsant effects [18].

We have previously shown that terpenoid derivatives from *Ferula* species have antileishmanial [19] and cancer chemopreventive [20,21] activities and can reverse multidrug resistance of cancer cells [22–25].

In present study, monoterpenoid stylosin was isolated from the roots of *Ferula ovina* for the first time (Fig. 1), and evaluated for its possible cytotoxic and apoptosis inducing effects. The investigations were carried out on two different human cell lines; 5637 cells (a TCC subline) and HFF3 cells (human fetal fibroblast). To study the cytotoxic activity of
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2. Materials and methods

2.1. Stylosin preparation

*F. ovina* was collected from Binaloud Mountains, 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. Dichloromethane extract of *F. ovina* roots (500 g) was obtained using maceration, and concentrated by solvent evaporation. The yield of extract was 18.6% (93 g). A part of this extract (20 g) was subjected to normal phase column (60×5 cm) chromatography. The elution of the column was started by petroleum ether and continued by increasing the amounts of ethyl acetate [petroleum ether:ethyl acetate, 20:1 (840 ml), 19:1 (1000 ml), 18:1 (950 ml), 17:1 (900 ml), 16:1 (850 ml), 14:1 (750 ml), 12:1 (650 ml), 10:1 (1100 ml), 5:1 (1200 ml) and 0:1 (2000 ml)]. 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 1, assigned as stylosin (706 mg, 3.5% of total extract), was a monoterpene derivative and its structure was confirmed by 1D- and 2D-Nuclear Magnetic Resonance (NMR) spectra. Its spectral data were in agreement with those previously described in the literature [29,30].

2.2. Preparation of various concentrations

To prepare different concentrations of stylosin (10, 20, 30, 40, 50, 60 and 70 μg/ml), 2 mg of the powder was dissolved in 500 μl dimethylsulfoxide (DMSO, Merck, Germany) and diluted with complete culture medium before experiments. In order to exclude the background cytotoxic effects of the solvent, the control treatments were also run with DMSO only. These controls included 0.25%, 0.5%, 0.75%, 1%, 1.25%, 1.5%, and 1.75% DMSO, equivalent to the DMSO content of their stylosin solutions.

Vincristine, vinblastine and cisplatin (Sobhan, Iran) were considered as positive controls in this study. Different concentrations of vincristine (25, 50, 100 and 250 μg/ml), vinblastine (25, 50, 100 and 400 μg/ml) and cisplatin (2, 10, 20, 50 and 100 μg/ml) were prepared using complete culture medium right before experiments.

2.3. Culture and growth of the cells

5637 cells were obtained from Pasteur Institute (Tehran, Iran) and HFF3 cells were a generous gift from Royan Institute (Tehran, Iran). All cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Scotland) supplemented with 10% fetal bovine serum (FBS, Gibco, Scotland) and incubated at 37°C in a humidified atmosphere of 5% CO2 in air. In order to subculture the cells, 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) were used for 3–5 min.

2.4. In vitro cytotoxicity assay

The thiazolyl blue (MTT) method [31] has been used in many studies to assess the cytotoxic effects of tested agents. The reduction of tetrazolium dye, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, by living cells is used in a rapid drug-screening assay. Briefly, cells were seeded at a density of 8 × 104 cells/well in 96-well tissue culture plates (Falcon Becton-Dickinson, USA). To identify the half maximal inhibitory concentration (IC50) of stylosin, 5637 and HFF3 cells were then incubated with increasing concentrations of stylosin (10, 20, 30, 40, 50, 60 and 70 μg/ml) for 24, 48 and 72 h, separately. The IC50 values of vincristine, vinblastine and cisplatin were also determined after incubating 5637 cells with increasing concentrations of vincristine (25, 50, 100 and 250 μg/ml) vinblastine (25, 50, 100 and 400 μg/ml) and cisplatin (2, 10, 20, 50 and 100 μg/ml) for 24, 48 and 72 h.

5 mg MTT dye (Sigma, Germany) was dissolved in 1 ml phosphate buffered saline (PBS), filtered and used freshly before each experiment. 20 μl MTT solution was then added to each well and plates were incubated for 4 h at 37°C. After this period, the blue insoluble formazan, produced from yellow MTT by living cells, was dissolved in DMSO (200 μl/well) during 2–3 min. The optic density (OD) of each well was then measured spectrophotometrically at 570 nm using an ELISA plate reader (Awareness, USA). All tests were performed in triplicates. Since the formation of formazan is correlated with the number of living cells, the OD read-outs from the treated wells were converted to the percentage of living cells against controls. This was calculated by dividing the absorbance of treated cells in each well to the mean absorbance of control cells.

2.5. Morphological alterations

5637 and HFF3 cells were cultured with various concentrations of stylosin (10, 20, 30, 40, 50, 60 and 70 μg/ml) and their DMSO controls. The cells were then observed under an invert microscope (Olympus, Japan) for morphological alterations 24, 48 and 72 h after their treatments.

2.6. Alkaline comet assay

In order to detect possible lesions of DNA, the alkaline version of the comet assay, which is a highly sensitive and
reliable biochemical method [32], was performed. Briefly, untreated 5637 cells, cells incubated with 37 μg/ml stylosin for 3 h and cells treated with its equivalent amount of DMSO (0.9%) were trypsinised and centrifuged at 3000 rpm (Orto Alresa Digicen 20, Spain) for 15 min.

After suspending cell pellets in 25 μl PBS, 75 μl of the 0.75% (w/v) low melting point agarose (LMA, Fermentas, Germany) was added. The cell suspensions were then dispensed onto microscope slides precoated with 1% (w/v) normal melting agarose (Helicon, Russia), and kept for 20 min at 4 °C. Slides were then covered with 100 μl of 0.75% (w/v) LMA and kept for another 20 min at 4 °C. Four slides were prepared for each sample. After immersing slides in freshly prepared lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 2% (v/v) triton X-100, pH 10) and incubating for 4 h at 4 °C, they were placed in an electrophoresis chamber filled with freshly prepared cold alkaline electrophoresis buffer (1 mM EDTA, 0.3 N NaOH, pH 13) and incubated for 30 min at 4 °C. Electrophoresis was conducted at 4 °C for 20 min in an electric field of 300 mA and 25 V. Slides were then washed with ice-cold neutralising buffer (0.4 M Tris–HCl, pH 7.5), dried with 96% ethanol, stained with ethidium bromide (20 μg/ml), and analysed using a fluorescent microscope (Olympus, Japan) attached to a CCD camera. Fifty cells per slide were evaluated and the mean of comet tail moment (a product of fraction of DNA in tail and tail length) was determined using TriTek Cometscore version 1.5 software. The DNA damage was expressed as % tail DNA, where it equals [tail DNA/(head DNA + tail DNA)] × 100.

2.7. Demonstration of apoptotic morphology

To assess the apoptotic effects of stylosin semi-quantitatively, 5637 cells were stained with 4′, 6-diamidino-2-phenylindole (DAPI), which selectively binds to the minor grooves of double stranded DNA. To do so, untreated 5637 cells, cells incubated with 37 μg/ml stylosin for 3 h and cells treated with its equivalent amount of DMSO (0.9%) were washed with PBS and fixed in 4% paraformaldehyde (Sigma, Germany). Cells were then washed with PBS and centrifuged at 1500 rpm (Orto Alresa Digicen 20, Spain) for three times, permeabilised with 0.1% triton X-100 and stained with 2 μg/ml DAPI (Merck, Germany) at 37 °C for 10 min. About 700 cells from each treatment were examined and counted under fluorescent microscope (Olympus, Japan), while chromatin condensation was the criteria used to demonstrate apoptosis.

Fig. 2. Time-based dose response curves of 5637 (A) and HFF3 (B) cells to stylosin during 24, 48 and 72 h. Data are expressed as mean ± SEM.
Fig. 3. Time-based dose response curves of 5637 cells to vincristine (A), vinblastine (B) and cisplatin (C) during 24, 48 and 72 h. Data are expressed as mean±SEM.
2.8. Statistical analysis

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

3. Results and discussion

In the present study, stylosin was isolated from the roots of *F. ovina* by normal phase column chromatography for the first time and its structure was confirmed by 1D- and 2D-NMR. To identify the IC_{50} of stylosin on 5637 cells, they were treated with various concentrations of stylosin for 24, 48 and 72 h; the viability of cells in each treatment was then compared with its relevant control, which was prepared using equivalent amounts of DMSO. Analyses of cell survival by MTT assay showed that the IC_{50} values of stylosin on 5637 cells were 37, 35 and 31 μg/ml after 24, 48 and 72 h of its administration, respectively (Fig. 2-A).

To compare this cytotoxic efficiency with the effects of known therapeutic reagents, 5637 cells were also treated with different concentrations of vincristine, vinblastine and cisplatin for the same period. The IC_{50} values of vincristine were identified as 70 μg/ml after 24 h and 50 μg/ml after 48 and 72 h of its administration, and the IC_{50} of vinblastine was 80 μg/ml after 48 and 72 h after its administration, while the IC_{50} values of cisplatin were identified as 12 μg/ml after 24 h and 8 μg/ml after 48 and 72 h of its administration (Fig. 3-A–C). These data indicate that the IC_{50} of stylosin on 5637 cells is less than both vincristine and vinblastine, but more than cisplatin during the same period of time.

To assess the cytotoxic effects of stylosin on non-cancerous cells, HFF3 cells were used. The IC_{50} values of stylosin were determined as 50, 39 and 38 μg/ml on HFF3 cells, after 24, 48 and 72 h of its administration on these cells, respectively (Fig. 2-B).

Morphological observations revealed that in comparison with untreated and control cultures, prominent cytoplasmic granulations were common on 5637 and HFF3 cells after treating with 37 and 50 μg/ml stylosin, respectively. Interestingly, only 3 h after the administration of stylosin, a large number of both normal and cancerous cell types became granulated and detached from the flasks (Fig. 4).

In order to determine the mechanism involved in cytotoxic effects of stylosin, DNA damage and chromatin condensation were analysed by alkaline version of comet assay and DAPI staining, respectively. Based on MTT results and morphological alterations, comet assay was carried out on untreated 5637 cells and cells incubated with 0.9% DMSO (as controls) and 5637 cells treated with 37 μg/ml stylosin (as test) for 3 h. Fig. 5 (A–C) represent photomicrographs of DNA lesions induced by 37 μg/ml stylosin and its relevant controls. Statistical analysis revealed that 37 μg/ml stylosin induced approximately 65% DNA damage, significantly (P<0.001) higher than that induced by 0.9% DMSO (12%, Fig. 5-G).

Fig. 4. Phase contrast photomicrographs of 5637 (A–C) and HFF3 (D–F) cells without any treatment (A and D), treated with 0.9% (B) and 1.25% (E) DMSO and treated with 37 μg/ml (C) and 50 μg/ml stylosin (F) for 3 hours.
Furthermore, demonstrating apoptotic morphology by DAPI staining revealed that 89% of cells treated with 37 μg/ml stylosin presented condensed chromatin, which was significantly \((P<0.001)\) higher than control (9%) and untreated (4%) cells (Fig. 5D–F).

In spite of major advances in cancer therapy, many malignancies including bladder carcinomas remain resistant to current chemotherapies \([33–35]\). This drawback has prompted a good deal of investigations in search for new compounds with antitumour properties.

In search for natural anticancer agents from edible plants, stylosin extracted from \(F. \textit{ovina}\) was subjected to cytotoxic screening program in this study. Our results revealed that the \(IC_{50}\) values of stylosin on 5637 cells were less than those of very well known drugs of vincristine and vinblastine. However, the \(IC_{50}\) values of stylosin on 5637 cells were higher in comparison with cisplatin, a synthetic drug used for treatment of TCC in combination therapy. Furthermore, it was shown that stylosin induces its cytotoxic effects on cancerous 5637 cells in less concentrations compared with normal HFF3 cells. It is interesting to mention that the cytotoxic effects of stylosin on both cell lines were observed just 3 h after its administration and increased during three continuous days.

In order to determine the mechanism involved in these effects, alkaline comet assay and DAPI staining were used. The results revealed that, in comparison with control (0.9% DMSO) cultures, 37 μg/ml stylosin significantly \((P<0.001)\) increased both DNA damage and chromatin condensation in 5637 cells, which are in agreement with morphological observations and MTT results.

Vanillic acid is a natural edible benzoic acid derivative which has been shown to possess antifilarial \([36]\), antimicrobial \([37]\), chemo preventive \([38]\), hepatoprotective \([39]\) and free radical scavenging \([40]\) effects. Analysing the...
composition of *F. ovina* root extract revealed that 3.5% of the total components were identified as stylosin. Since there are no reports indicating *in vivo* or *in vitro* cytotoxic effects of vanillic acid, it seems that stylosin induces its effects via its terpenoid moiety or perhaps the linkage of vanillic acid and borneol causes stylosin properties. The apoptotic effects of monoterpene compounds have been frequently reported, and these effects were proven to act through the up-regulation of p21/Cip1 [13], down-regulation of Bcl-2, cyclin D1 and cdk4 [7,13,41], lipid peroxidation [9] and activation of caspase-3 [14]. Further studies are needed to determine whether stylosin induces its cytotoxic effects similar to other monoterpene compounds or has different cellular and molecular activities. Moreover, changes in the chemical structure of stylosin may enhance its selective cytotoxicity on cancerous cells, which needs comprehensive structure-activity relationship studies.

4. Conclusion

*F. ovina* is an edible plant that has been widely used in Iranian traditional medicine for its great therapeutic properties [18]. Present results indicate that stylosin, a monoterpene extracted from the roots of *F. ovina*, has selective cytotoxic effects, since its IC50 values on cancerous cells (5637) were less than non-cancerous cells (HFF3). However, a comprehensive study is required to see if it has similar effects on other tumour cell lines and in vivo.

Since it is possible to synthesize stylosin and also extract it from the roots of *F. ovina* and other *Ferula* species, and due to its great (more than vincristine and vinblastine), quick (just after 3 h) and long-lasting (72 h) cytotoxic effects, stylosin can potentially be considered as an effective anticancer agent for future *in vivo* and clinical studies.

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