RESEARCH ARTICLE

Anti-proliferative and Apoptotic Effects of Dendrosomal Farnesiferol C on Gastric Cancer Cells

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Abstract

Farnesiferol C is a natural compound with various anti-cancer properties that belongs to the class of sesquiterpene coumarins. However, the low bioavailability of farnesiferol C limits its therapeutic potential. Here, we overcame this problem utilizing dendrosome nano-particles and evaluated the anti-cancer effect of dendrosomal farnesiferol C (DFC) on the AGS gastric cancer cell line. The 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and reverse transcriptase-polymerase chain reaction (RT-PCR) were respectively used to detect the anti-proliferative properties of DFC and expression ratio of Bax/Bcl-2 as a hallmark of apoptosis. Compared to the void farnesiferol C (FC), our data showed that DFC significantly suppresses the proliferation of AGS cells in a time- and dose-dependent manner (P<0.01). Also, DFC meaningfully increased the expression ratio of Bax/Bcl-2 in AGS cells (P<0.01). The findings demonstrate that our nano-based formulation of farnesiferol C could be considered as a potential therapeutic agent in cancer targeting.

Keywords: AGS cell line - Bax/Bcl-2 - coumarin - dendrosome - phytochemicals

Asian Pac J Cancer Prev, **16 (13)**, 5325-5329

Introduction

Nowadays, cancer is considered as a common cause of morbidity and mortality in the world (Soltanzade et al., 2012). According to the World Health Organization (WHO), cancer is the third most common cancer worldwide with a frequency that varies greatly across the different geographic locations (Bozzetti et al., 1999). Current therapies for gastric cancer are surgery, chemotherapy and radiotherapy (Watanabe et al., 2010). However, these remedies are often ineffective (Hoshyar et al., 2013) and the 5-year overall survival rate is 25.7%, which has not changed drastically over the past 30 to 40 years (Kumar et al., 2013). Recently, the limited achievements and significant side effects of Gastric cancer treatments with classic chemotherapeutic agents have led to the huge amounts of scientific efforts to re-focus on traditional medicine as a rich resource of the effective phytochemicals with lower side effects (Garodia et al., 2007). Coumarin is a fragrant natural compound with a colorless crystalline substance in its standard state and could be found in different variety of plants. Coumarins consist of a large class of phenolic materials containing 2*H*-1-benzopyran-2-one (called coumarin) moiety that can be synthesized by numerous green plant species (Elinos-Baez et al., 2005; Venugopala et al., 2013; Kundu and Chun, 2014). Although, lots of different cytotoxic activities have been observed along the studies of coumarins (Rana et al., 2014), the mechanism of actions has not been fully understood yet. The rather low toxicity against normal cells versus cancerous cell lines has made coumarins as attractive candidates for the future anti-cancer medications (Lake et al., 1999). Among the large family of coumarins, the farnesiferol C (FC) is one of the sesquiterpene coumarin compounds that is isolated from the roots of *Ferula* szowitziana D.C (Shahverdi et al., 2007; Mashinchian et al., 2010). The plants of genus *Ferula* (Apiaceae) have distributed throughout central Asia, Mediterranean region and Northern Africa (Pimenov and Leonov, 1993) and well documented as a good source of bioactive compounds such as sesquiterpenes and coumarins (Zhou et al., 2000; Abd El-Razek et al., 2001; Iranshahi et al., 2004). Farnesiferol C shows a broad range of biological activities such as anti-leishmaniasis, anti-angiogenesis and anti-cancer effects (Ryu et al., 2004). However, the poor solubility and bioavailability of this phytochemical *in vivo* and *in vitro* have limited its exploitation as therapeutic agent (Mashinchian et al., 2010). We have demonstrated that by employing our dendrosome nano-particles *in vitro*, the solubility of FC thereby its anti-proliferation effect on cancer cells could be improved. Dendrosomes are neutral, covalent, self-assembled, biodegradable and spherical nano-particles and their exceptional capabilities have been shown in safely delivering genes and herbal compounds into the different cell lines (Sarbolouki et al., 2000; Sadeghizadeh et al., 2008; Mashinchian et al., 2010; Babaei et al., 2012, Dehghan Esmatabadi et al., 2015). Current work covers

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the study of anti-proliferation and apoptotic effects of DFC compared to FC. We evaluated the viability of AGS cells as well as the expression ratio of Bax/Bcl-2 genes, the latter as a hallmark of apoptosis.

Materials and Methods

Cells and reagents

AGS cell lines (human gastric carcinoma cells lines) were purchased from Pasteur Institute of Iran (Code: C131). Farnesiferol C (with more than 99% purity) (Figure 1) and dendrosome nanoparticles specified as Den O400 (a nonionic biodegradable denderic glycol ester with MW: 590 Da, HLB: 12.5 Mh/M, hydroxyl value: 95 mg KOH/g and acid value: mg KOH/g), were provided by Color Research Institute in Tehran.

Dendrosomal farnesiferol C preparation

To prepare a media soluble nano-compound of farnesiferol C, different weight/weight ratios of the phytochemical and dendrosome were tested for absorbance spectra by spectrophotometry in comparison with control samples including farnesiferol C dissolved in PBS and 1% methanol. Regarding the excitation/emission value, an appropriate proportion of 10:1 for dendrosome: farnesiferol C was settled and stored at 4ºC.

Cell culture

AGS cell lines were cultured in RPMI-1640 (Gibco, USA) containing 10% heat inactivated fetal bovine serum (FBS; Gibco, USA), 1% penicillin-streptomycin (Gibco, USA) at 37°C in a humidified atmosphere of 5% CO_2. For all of the experiments the cells in exponential growth were harvested using 0.25% Trypsin and their viability was determined by Trypan blue exclusion assay and as a rule, only cells with viability higher than 90% were used for treatments.

Cell viability assay

The cytotoxic effects of dendrosomal farnesiferol C were investigated on AGS cells by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay according to the manufacturer's instructions (Sigma-Aldrich, USA). AGS cells (10000 cells/well) were seeded onto 96-well plates in RPMI-1640 containing 10% FBS. The cells were allowed to attach for 24 h before treatment and then they were incubated with fresh medium containing serial concentrations (0 to 150 μM) of dendrosomal and void farnesiferol C for 24 and 48h. Untreated AGS cells were used as negative control in all experiments. After incubation at different time courses, 5 mg/ml MTT solution was added into each well, and the plates were incubated for 4h (37°C, 5% CO_2). Then, media containing MTT were removed and DMSO was added in each well to dissolve the formazan crystals. After 10 minutes of incubation at room temperature, absorbance was read at 570 nm by a 96-well plate reader (Tecan, Switzerland). Then, the concentration at which cell growth was inhibited by 50% (IC_{50}) was determined by standard curve method. Each experiment was carried out in triplicate and repeated at least three times.

RNA extraction and semi-quantitative RT-PCR reaction

Sample preparation: The exponentially growing AGS cells were seeded at a density of 2.5×10^5 cells/well in six well plates and allowed to adhere and grow overnight. Then, cells treated with 80 μM dendrosomal farnesiferol C for 24 and 48h. Untreated wells were also used as negative controls.

RNA extraction: All treated and untreated AGS cells were collected after 24 and 48h treatment and total RNA was extracted by using RNX-plus reagent (Cinnagen, Iran) according to the manufacturer's instructions. The quality and quantity of extracted RNAs were analyzed respectively by 1% agarose gel electrophoresis and Nano drop (Thermoscientific, USA).

Primer design: Primer sequences for β2m (as an internal control) were designed from published sequences in the GeneBank nucleic acid database (accession number: NM-004048) by using genrunner software (version 3.6) and synthesized by Microgen Co (South Korea). Furthermore, Bax and Bcl-2 primers were chosen from the previous report published by Karagozlua (Karagozlua et al., 2010).

Polymerase chain reaction (PCR): Complementary DNA (cDNA) was synthesized with the same amount of the extracted RNA (1.5μg) for all samples, 0.5μg Oligo-dT primer and RevertAidTMM-Mulv Reverse transcriptase according to the manufacturer's instruction (Invitrogen, USA).

Then, PCR reaction was performed to amplify Bax and Bcl-2 cDNAs in a final volume of 20μl on a thermal cycler (Techne Co.) using the following primer pairs:

Human Bax:

HBaF (as forward primer): 5 ´-TGC CAG CAA ACT GGT GCT CA- 3′ (417-436) and

HBaR (as reverse primer): 5 ´-GCA CTC CCG CCA CAA AGA TG- 3′ (610-591),

Human Bcl-2:

HBcF (as forward primer): 5 ´-CGC ATC AGG AAG GCT AGA GT- 3′ (2117-2136) and HBcR (as reverse primer): 5 ´-AGC TTC CAG ACA TTC GGA GA- 3′ (2305-2286), that amplified 194 bp and 189 bp segments from Bax and Bcl-2 cDNAs, previously.

PCR conditions for Bax and Bcl-2 cDNA amplification were as the following: pre-denaturation for 5 min at 95 °C; following by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 min.

Also, β2m cDNA was amplified with similar conditions of PCR for Bax and Bcl-2 except for 35 cycles of reaction. These primers amplified a 191 bp segment from β2m cDNA.

Quantification of PCR products: PCR products were separated by electrophoresis through a 1.5% agarose gel, stained with ethidium bromide, and then visualized under the UV light.

For semi-quantitative analysis of PCR bands, the density of each band was determined using Uvitec software (Uvitec, UK). To ensure data accuracy derived from UV transillumination during photography of the complementary DNA containing gel, the light output, color and contrast settings in software were considered identical.

Also, to ensure that the equal amounts of RNA were used for each reaction and that the potential differences in signal intensity were not due to differences in the amounts of starting RNA, β2m was used as an internal control for each reaction. RT-PCR was performed in separate tubes under similar conditions (except for the cycle number) for Bax, Bcl-2 and β2m. The results were expressed as the ratio of Bax and Bcl-2 gene bands' intensities to the intensity of β2m to calculate any differences in the starting amounts of RNA.

Statistical analysis

All experiments were replicated two or three times and data were expressed as mean ± standard deviation (SD). Database was set up with SPSS software version 16.0 and comparisons between different groups were done by One-Way Analysis of Variance (ANOVA). Student's t-test was used to compare the mean of each group with that of the control group. The P values less than 0.05 were also considered statistically significant.

Results

Effect of dendrosomal farnesiferol C and void farnesiferol C on AGS cells

We determined the toxicity of DFC and FC on AGS cancerous cell lines by MTT assay. As the results show in Figure 2, DFC has morphologically affected cancer cells. Regarding MTT assay, we demonstrated that DFC significantly suppresses the proliferation of AGS cells in a time- and dose-dependent manner (P<0.01) (Figure 3A).

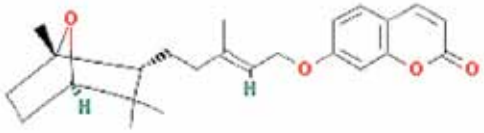

Figure 1. Chemical Structure of Farnesiferol C

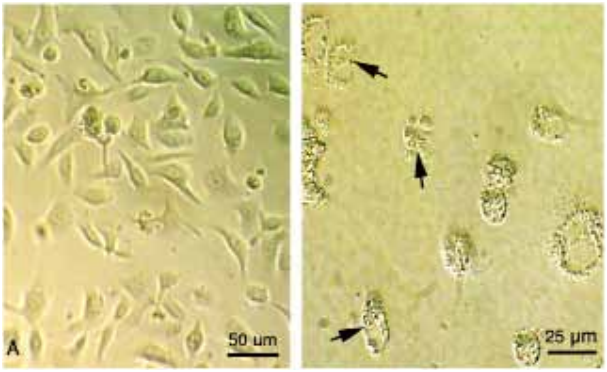

Figure 2. The Microscopic images of AGS Cells (A) Normal Cells (20× Magnification) (B) Treated Cells with 80 µM Dendrosomal Farnesiferol C (40× Magnification), Show Clear Changes in Cell Number and Appearance

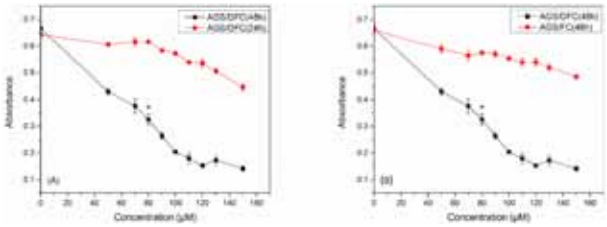

Figure 3. Effect of Dendrosomal Farnesiferol C and Void Farnesiferol C on the Viability of Human Adenocarcinoma Gastric Cell Line. AGS Cells were treated with different concentrations of DFC and FC for 24 and 48 h and were checked for their proliferation by MTT assay (A) DFC inhibits proliferation of AGS cells in a dose- and time-dependent manner (P<0.01) (B) DFC significantly (P<0.01) inhibits proliferation of AGS cells compared to void farnesiferol C. Data reported are mean±SD. DFC: Dendrosomal farnesiferol C, FC: farnesiferol C. * IC_{50} value of dendrosomal farnesiferol C that results in 50% of AGS cells proliferation inhibition

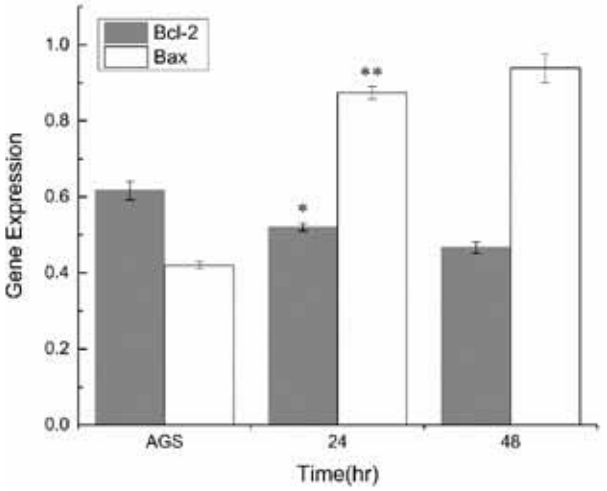

Figure 4. RT-PCR Analysis of Bcl-2 and Bax Genes in Three Different Groups, Including AGS Cells (As Control Group), AGS Cells Treated for 24 and 48h with 80 µM DFC. The data demonstrated that DFC upregulated the expression of Bax and downregulated the expression of Bcl-2 in the AGS cells (P<0.01), in a time dependent manner compared to the control cells. * and ** Indicates the significance of data at the level of P<0.01compared to the findings in control cells

Conversely, FC did not show any meaningful effect on cancer cells (Figure 3B).

The IC_{50} values for DFC within 24 and 48h were >150 µM and 80 µM, respectively. This value for FC was more than 150 µM for both times.

Detection of Bax and Bcl-2 gene expression in the AGS cells

The relative expression level of Bax and Bcl-2 to β2m was evaluated in treated and untreated AGS cells by RT-PCR. As shown in Figure 4, the expression of Bax gene has increased in treated samples compared to the untreated cells (P<0.01). Conversely, the expression of Bcl-2 has decreased in treated samples.

Additionally, statistical analysis demonstrates that the expression ratio of Bax/Bcl-2, as a hallmark of apoptosis, has significantly increased in DFC treated cells in a time dependent manner.

Discussion

Anti-cancer agents have been divided into two broad categories according to their cytotoxic and/or anti-proliferative abilities (Keskin et al., 2000). Sesquiterpene coumarin compounds isolated from the Apiaceae genus, have been shown both of the growth inhibitory and cytotoxic activities in different cancerous cell lines (Ryu et al., 2004).

Umbelliprenin is a prenylated compound that belongs to the class of sesquiterpene coumarins and exerts anti-proliferative effects on M4Beu cells (human metastatic pigmented malignant melanoma cell line) through cell cycle arrest in G1 phase (Barthomeuf et al., 2008) and cytotoxic effects on A549 (human lung cancer cell line) via mitochondrial dependent mechanisms (Barthomeuf et al., 2008; Khaghanzadeh et al., 2012). However, cytotoxic effects of Umbelliprenin have been observed selectively in malignant cells (Ryu et al., 2004; Khaghanzadeh et al., 2012; Paydar et al., 2013).

Farnesiferol C of the sesquiterpene coumarin compounds showed potent cytotoxic and inhibitory activities in various human tumors, including A549, SK-OV-3 (human ovary cancer cell line), SK-MEL-2 (human melanoma cancer cell line) (Ryu et al., 2004).

As one of the most challenging tasks concerning cancer therapy is to induce apoptosis in malignant cells, researchers increasingly focus on the natural products to modulate apoptotic signaling pathways (Liu et al., 2014; Khairi et al., 2014; Zhang et al., 2014; Tawil et al., 2015).

Apoptosis (programmed cell death) regulates tissue homeostasis in animals that in mammalian cells has two major pathways: the intrinsic or mitochondrion-mediated and the extrinsic or death receptor-mediated pathways, both of which result in the activation of different caspases (Fesik, 2005). The Bcl-2 family of intracellular proteins are the central regulator of caspase activations, and divided into pro-apoptotic (like Bax) and anti-apoptotic (like Bcl-2) proteins. Pro-apoptotic and anti-apoptotic proteins interact with each other to determine the survival or commitment to apoptosis in cells (Frenzel et al., 2009; Chipuk et al., 2010; Gholami et al., 2013).

In spite of the potent anti-tumor effect of farnesiferol C against a great variety of cancer cell lines, the poor solubility of FC in aqueous solutions and consequently its low bioavailability have limited its application as a medicine (Ryu et al., 2004; Mashinchian et al., 2010).

In this study, we report that dendrosomal farnesiferol C at high concentrations (>50 μM), significantly affects viability of AGS cells in a time- and dose-dependent manner compared to FC. These data suggest that dendrosome nanoparticles significantly improve the solubility of FC, increase its uptake in cultured cells and enhance cytotoxicity. Based on our previous works that introduced dendrsomes as a safe gene/drug porter *in vitro* and in (Sarbolouki et al., 2000; Sadeghizadeh et al., 2008; Babaei et al., 2012), here we confirmed this characteristic for Farnesiferol C as a potent herbal remedy.

To find out somewhat the molecular aspect of cytotoxic effect of DFC on AGS cells, we evaluated the expression ratios of Bax/Bcl-2 genes as a hallmark of apoptosis.

RT-PCR analysis of Bax and Bcl-2 genes showed that DFC could suppress AGS cell proliferation via in part inducing apoptosis. These observations are in concordance with other recent reports that coumarin could induce apoptosis by modulating Bax/Bcl-2 and caspase pathways (Sadeghizadeh et al., 2008; Gholami et al., 2013).

In conclusion, our primary results demonstrate that the nano-based formulation of farnesiferol C (DFC) offers a great potential as an appropriate candidate within herbal compounds with anti-cancer properties that its anti-cancer effects is partly via inducing the tumor cell apoptosis by increasing the Bax/Bcl-2 expression ratio. With regard to its solubility and stability in the medium compared to the void farnesiferol C, it is suggested as an anticancer agent against stomach cancer in humans. However, further investigations should be done to…

Acknowledgements

Authors would like to thank the University of Tabriz for financial and technical support of this research project.

References

Abd El-Razek MH, Ohta S, Ahmed AA, et al (2001). Sesquiterpene coumarins from the roots of Ferula assa-foetida. *Phytochemistry*, **58**, 1289-95.

Babaei E, Sadeghizadeh M, Hassan ZM, et al (2012). Dendrosomal curcumin significantly suppresses cancer cell proliferation *in vitro* and *in vivo*. *Int Immunopharmacol*, **12**, 226-34.

Barthomeuf C, Lim S, Iranshahi M, et al (2008). Umbelliprenin from Ferula szowitsiana inhibits the growth of human M4Beu metastatic pigmented malignant melanoma cells through cell-cycle arrest in G1 and induction of caspase-dependent apoptosis. *Phytomedicine*, **15**, 103-11.

Bozzetti F, Marubin EI, Bonfanti G, et al (1999). Subtotal versus total gastrectomy for gastric cancer: five-year survival rates in a multicenter randomized Italian trial. *Ann Surg*, **230**, 170-8.

Chipuk JE, Moldoveanu T, Llambi F, et al (2010). The BCL-2 family reunion. *Mol cell*, **37**, 299-310.

Dehghan Esmatabadi MJ, Farhangi B, Safari Z, et al (2015). Dendrosomal curcumin inhibits metastatic potential of human SW480 colon cancer cells through down-regulation of Claudin1, Zeb1 and Hef1-1 Gene Expression. *Asian Pac J Cancer Prev*, **16**, 2473-81.

Elinos-Baez C, Leon F, Santos E (2005). Effects of coumarin and 7OH-coumarin on bcl-2 and Bax expression in two human lung cancer cell lines *in vitro*. *Cell Biol Int*, **29**, 703-8.

Fesik SW (2005). Promoting apoptosis as a strategy for cancer drug discovery. *Nat Rev Cancer*, **5**, 876-85.

Frenzel A, Grespi F, Chmelewskij W, et al (2009). Bcl2 family proteins in carcinogenesis and the treatment of cancer. *Apoptosis*, **14**, 584-96.

Garodia P, Ichikawa H, Malani N, et al (2007). From ancient medicine to modern medicine: ayurvedic concepts of health and their role in inflammation and cancer. *J Soc Integr Oncol*, **5**, 25-37.

Gholami O, Jeddi-Tehrani M, Iranshahi M, et al (2013). Umbelliprenin from ferula szowitsiana activates both intrinsic and extrinsic pathways of apoptosis in Jurkat T-CLL cell line. *Iran J Pharm Res*, **12**, 371-6.

Hoshyar R, Bathaie SZ, Sadeghizadeh M (2013). Crocin triggers the apoptosis through increasing the Bax/Bcl-2 ratio and

caspase activation in human gastric adenocarcinoma, AGS, cells. *DNA Cell Biol*, **32**, 50-7.

Iranshahi M, Amin G, Shafiee A (2004). A new coumarin from Ferula persic. *Pharml boil*, **42**, 440-2.

Karagozlua MZ, Kima JA, Karadeniza F, et al (2010). Anti-proliferative effect of aminoderivatized chitooligosaccharides on AGS human gastric cancer cells. *Process Biochemistry*, **45**, 1523-8.

Keskin O, Bahar I, Jernigan R, et al (2000). Characterization of anticancer agents by their growth inhibitory activity and relationships to mechanism of action and structure. *Anticancer Drug Des*, **15**, 79-98.

Khaghanzadeh N, Mojtahedi Z, Ramezani M, et al (2012). Umbelliprenin is cytotoxic against QU-DB large cell lung cancer cell line but anti-proliferative against A549 adenocarcinoma cells. *Daru*, **20**, 69.

Khairi M, Shamsuddin S, Jaafar H, et al (2014). Effects of rapamycin on cell apoptosis of MCF-7 human breast cancer cells. *Asian Pac J Cancer Prev*, **15**, 10659-63.

Kumar RK, Raj SS, Shankar EM, et al (2013). Gastric Carcinoma: A Review on Epidemiology, Current Surgical and Chemotherapeutic Options. *InTec*, 273-293, (DOI: 10.5772/52138).

Kundu JK, Chun KS (2014). The promise of dried fruits for cancer chemoprevention. *Asian Pac J Cancer Prev*, **15**, 3343-52.

Lake B (1999). Coumarin metabolism, toxicity and carcinogenicity: relevance for human risk assessment. *Food Chem Toxical*, **37**, 423-53.

Liu YQ, Li Y, Qin J, et al (2014). Matrine reduces proliferation of human lung cancer cells by inducing apoptosis and changing miRNA expression profiles. *Asian Pac J Cancer Prev*, **15**, 2169-77.

Mashinchian O, Salehi R, Dehghan G, et al (2010). Novel thermosensitive poly (N-isopropylacrylamide-co-vinylpyrrolidone-co-methacrylic acid) nanosystems for delivery of natural products. *Int J Drug Delivery*, **2**, 278-86.

Paydar M, Wong YL, Moharam BA, et al (2013). Pharmacological Activities and Chemical Constituents of Ferula szowitsiana DC. *Int J Med Sci*, **13**, 236-43.

Pimenov MG, Leonov M (1993). The genera of the Umbelliferae: a nomenclator. *Edinburgh J Botany*, **52**, 92-3

Rana A, Altar R, Qureshi MZ, et al (2014). Dealing naturally with stumbling blocks on highways and byways of TRAIL induced signaling. *Asian Pac J Cancer Prev*, **15**, 8041-6

Ryu SY, Lee CO, Choi SU, et al (2004). Anticancer composition comprising sesquiterpenes isolated from resina ferulae. Google Patents. Available from https://www.google.com/patents/US20040043083.

Sadeghizadeh M, Ranjbar B, Damaghi M, et al (2008). Dendrosomes as novel gene porters-III. J *Chem Technol Biotechnol*, **83**, 912-20.

Sarbolouki MN, Sadeghizadeh M, Yaghoobi MM, et al (2000). Dendrosomes: a novel family of vehicles for transfection and therapy. *J Chem Technol Biotechnol*, **75**, 919-22.

Shahverdi AR, Fakhimi A, Zarrini G, et al (2007). Galbanic acid from Ferula szowitsiana enhanced the antibacterial activity of penicillin G and cephalexin against Staphylococcus aureus. *Biol Pharm Bull*, **30**, 1805-07.

Soltanzade SS, Barar J, Nazemyieh H, et al (2012). Evaluation of cytotoxicity and anti-cancer effect of Ferula szowitsiana methanolic extract on lung cancer A549 cell-lines. *Res Pharm Sci*, **7**, 103.

Tawil M, Bekdash A, Mroueh M, et al (2015). Wild carrot oil extract is selectively cytotoxic to human acute myeloid leukemia cells. *Asian Pac J Cancer Prev*, **16**, 761-7.

Venugopala KN, Rashmi V, Odhav B (2013). Review on natural coumarin lead compounds for their pharmacological activity. *Biomed Res Int*, **2013**, 1-14.

Watanabe T, Kume K, Tai M, et al (2010). Gastric mucosal cancer smaller than 7 mm can be treated with conventional endoscopic mucosal resection as effectively as with endoscopic submucosal dissection. *Hepato-Gastroenterol*, **57**, 668-73.

Zhang N, Wang D, Zhu Y, et al (2014). Inhibition effects of lamellarin D on human leukemia K562 cell proliferation and underlying mechanisms. *Asian Pac J Cancer Prev*, **15**, 9915-9

Zhou P, Takaisha Y, Duan H, et al (2000). Coumarins and bicoumarin from Ferula sumbul: anti-HIV activity and inhibition of cytokine release. *Phytochemistry*, **53**, 689-97.