



Drimane-type Sesquiterpene Coumarins from *Ferula gummosa* Fruits Enhance Doxorubicin Uptake in Doxorubicin-resistant Human Breast Cancer Cell Line

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ABSTRACT

Multidrug resistance (MDR) is the main cause of failure in the chemotherapy of cancer patients. The present study aimed to evaluate the effects of sesquiterpene coumarins of *Ferula gummosa* fruits on P-glycoprotein (P-gp)-mediated MDR. Drimane-type sesquiterpene coumarins from the fruits of *F. gummosa* were extracted with dichloromethane and subjected to column chromatography. The effects of the isolated compounds on P-gp-mediated MDR were evaluated in the breast cancer cell line MCF-7 which shows high resistance to doxorubicin (MCF-7/Dox). Phytochemical investigation of dichloromethane extract of *F. gummosa* fruits resulted in three sesquiterpene coumarins including conferone (1), mogoltacin (2), and feselol (3). The structures of these compounds were confirmed by 1D and 2D Nuclear Magnetic Resonance (NMR) spectroscopy. Exposure of cells to conferone, mogoltacin, feselol, and verapamil (positive control) enhanced doxorubicin uptake by MCF-7/Dox cells. This effect was dose dependent, but varied with the structure of the chemical. At 25 μ M, all the tested sesquiterpene coumarins restored at least 50% of the reference uptake (uptake by sensitive cells); but at 10 μ M, their potency varied where conferone showed the highest potency and feselol showed the lowest potency. Conferone, mogoltacin, and feselol from *F. gummosa* suppress P-gp-mediated drug efflux in highly resistant human breast cancer cells.

Key words: Breast cancer, *Ferula gummosa*, P-glycoprotein inhibitor, Sesquiterpene coumarins

INTRODUCTION

Multidrug resistance (MDR) is the main cause of failure in chemotherapy of cancer patients. Although MDR is a multifactorial

phenomenon, its predominant cause is the overexpression of membrane transporter proteins belonging to the ATP-binding cassette superfamily, namely P-glycoprotein (P-gp; ABCB1) and multidrug resistance protein (MRP1 or ABCC1).^[1,2]

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The observation that many compounds of natural origin are capable of modulating MDR proteins has called increasing attention to these substances. Recent reports have demonstrated that sesquiterpene coumarins, which contain drimane-type structure in the sesquiterpene moiety, reverse P-gp-mediated resistance considerably.^[3,4] This class of natural compounds has been especially reported from the genus *Ferula* (Apiaceae).^[5] Many bioactive compounds have been reported from *Ferula* species,^[6-9] but the biological activities of drimane-type sesquiterpene coumarins have poorly been documented.^[3,4,10]

Objectives

In the present work, we isolated the sesquiterpene coumarins of *Ferula gummosa* fruits and evaluated the effects of three of them (conferone, mogoltacin, and feselol) on P-gp-mediated MDR in the breast cancer cell line MCF-7 which shows high resistance to doxorubicin (MCF-7/Dox). The tested compounds belong to drimane-type sesquiterpene coumarins.

MATERIALS AND METHODS

Plant material

Fruits of *F. gummosa* were collected from Hezar Masjed Mountains, northeast of Iran, in August 2005 and identified by Mohammadreza Joharchi at the Ferdowsi University of Mashhad Herbarium (FUMH). A voucher specimen (1002) was deposited at the herbarium of the Department of Pharmacognosy, School of Pharmacy, Mashhad University of Medical Sciences.

General experimental procedures

The ¹H, gradient CORrelation Spectroscopy (gCOSY), Rotating-frame Overhauser Effect Spectroscopy (ROESY), gradient Heteronuclear Single Quantum Coherence (gHSQC), and gradient Heteronuclear Multiple Bond Coherence (gHMBC) Nuclear Magnetic Resonance (NMR) experiments were run under standard conditions on Bruker DRX-500 and DRX-600 spectrometers at 300 K. NMR samples were prepared by dissolving each sample in CDCl₃ (99.8% D) (Carlo Erba, Italy). The spectra were calibrated using the solvent signal as the internal standard (¹H, δ: 7.27 ppm; ¹³C, δ: 77.0 ppm). The ROESY spectra were obtained with a mixing time of 400 ms. The NMR data were processed on a Silicon Graphic Indigo2 Workstation using UXNMR software. Column chromatography was conducted with silica gel 230-400 mesh (Merck, Germany).

Extraction and isolation

The air-dried fruits (500 g) were ground into powder, defatted by petroleum ether, and extracted exhaustively by maceration with dichloromethane at room temperature. After filtration, the extract was concentrated under vacuum to yield 20 g of a brown residue.

A part of the extract (15 g) was subjected to column chromatography on silica gel (5 × 50 cm) using petroleum ether with increasing volumes of acetone in a gradient system. The fractions were compared by thin layer chromatography (TLC; silica gel using petrol–Me₂CO as solvent), and those giving similar spots were combined. Five fractions were finally obtained. Fractions 1-3 gave compounds 1 (15 mg), 2 (770 mg), and 3 (48.5 mg) as white crystals, respectively.^[11]

Cell line and P-gp inhibition assay

Resistant MCF-7/Dox cells were generated by one year of continuous exposure of sensitive MCF-7 cells (European Collection of Cell Cultures, Salibury, UK) to Dox. These cells have been shown to overexpress P-gp. Based on a previously described procedure,^[12] cells were cultured as monolayers in 75-cm² culture flasks in Eagle's minimum essential medium (Gibco-BRL, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS; Bio West, Nuaille, France), vitamins, amino acids, and gentamycin (all from Gibco™, Invitrogen, Cergy-Pontoise, France). The medium was changed every 48 h and subcultured with 5X trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco). Cells were maintained at 37°C under a humidified atmosphere containing 5% CO₂. The chemicals were obtained from Sigma, USA.

Cytotoxicity was assessed using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay. Briefly, the cells were seeded in 96-well tissue culture plates and incubated with increasing concentrations of each compound (10, 20, 30, 40, 50, 60, and 70 μg/ml) for 24 h. Twenty microliters of MTT in phosphate-buffered saline (PBS; 5 mg/ml) was then added to each well. Following a 4-h incubation at 37°C, the medium/MTT mixtures were removed and the blue insoluble formazan product formed by living cells was dissolved in dimethyl sulfoxide (DMSO) (200 μl/well). The optical density of the wells was then measured with a microplate reader at 570 nm. All tests were performed in triplicate and the 50% inhibitory concentrations (IC₅₀) were calculated from concentration–effect curves by interpolation.

Cellular Dox accumulation was determined as described by Huang and Liu.^[13] In brief, MCF-7/Dox cells in exponential growth were exposed to 10 μM doxorubicin in the absence or presence of conferone, mogoltacin, and feselol at 10 and 25 μM or verapamil (VPM; Sigma) at 10 μM for 3 h. Then the cells were harvested by centrifugation and rinsed with PBS. Cell pellets were suspended in 0.3 μM HCl in 50% ethanol and sonicated in an ultrasonic disintegrator for 30 s. Following centrifugation at 1500 g for 15 min, the supernatant was removed and assayed spectrofluorometrically for Dox content at excitation and emission wavelengths of 470 nm and 585 nm, respectively. The intracellular Dox content was calibrated with a standard curve of Dox and was expressed in picomoles per 10⁶ cells.

Statistical analysis

Statistical analyses were performed using the SPSS (version 11.0) software. Statistical comparison between groups was made by Student's *t*-test and one-way analysis of variance (ANOVA) with Tukey–Kramer, a *post-hoc* test. A *P* < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Silica gel column chromatography of the dichloromethane extract of fruits of *F. gummosa* followed by preparative TLC resulted in three sesquiterpene coumarins including conferone (1), mogoltacin (2), and feselol (3), as reported in an earlier study.^[11] The isolated coumarins [Figure 1] were identified by comparison of their NMR, mass spectrometry (MS), and melting point data

with those previously described in the literature.^[5,14,15] Briefly, the structure of 1 was established from analysis of the ¹H and ¹³C NMR spectra [Tables 1 and 2]. Compound 1 displayed 24 carbon

signals, with 9 being typical of an umbelliferone skeleton and the other 15 signals were ascribable to a sesquiterpene moiety. The downfield signal at δ_c 161.5 was assigned to the carbonyl carbon

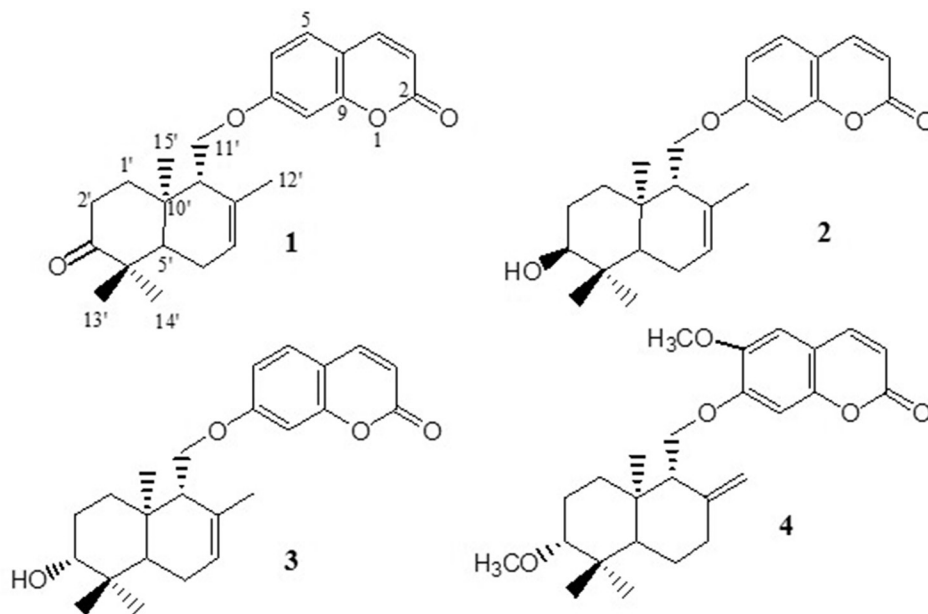


Figure 1. Chemical structures of the compounds isolated from *Ferula gummosa* fruits conferone (1), mogoltacin (2) and feselol (3) and the structure of diportlandin (4)

Table 1. ¹H-NMR data obtained with compounds 1-5 (600 MHz, δ ppm)*

Position of proton	1 (J)	2 (J)	3 (J)	4 (J)	5 (J)
2	-	-	-	-	-
3	6.28 d (9.6)	6.27 d (9.5)	6.27 d (9.5)	6.30 d (9.5)	6.25 d (9.5)
4	7.66 d (9.6)	7.66 d (9.5)	7.66 d (9.5)	7.67 d (9.5)	7.65 d (9.5)
5	7.39 d (8.4)	7.38 d (8.4)	7.39 d (8.4)	7.39 d (8.4)	7.39 d (8.4)
6	6.86 dd (8.4, 2.3)	6.86 dd (8.4, 2.3)	6.88 dd (8.4, 2.3)	6.87 dd (8.4, 1.8)	6.83 d (8.4)
7	-	-	-	-	-
8	6.85 d (2.3)	6.85 d (2.3)	6.85 d (2.3)	6.86 d (1.8)	6.82 brs
9	-	-	-	-	-
10	-	-	-	-	-
1'	1.66 dd* β 2.30 m α	1.75 m* β 2.01 m* α	1.37 dd (13.2, 4.2) β 2.05 m* α	1.68 m β 2.01 m α	1.55 ddd (15.0, 6.0, 6.0) β 1.97 dt (15.0, 4.2) α
2'	2.31 m β 2.75 ddd (15.3, 6.6) α	1.69 m	1.67 m	1.70 m β 2.00 m α	1.69 m
3'	-	3.52 brs	3.31 brd (10.8)	3.41 brs	3.23 dd (10.8, 4.2)
4'	-	-	-	-	-
5'	1.68 dd*	1.73 dd*	1.30 dd (11.4, 6.0)	2.67 s	2.16 s
6'	2.00 like brd β 2.18 like brt α	2.0 m*	2.01 m*	-	-
7'	5.62 brs	5.59 brs	5.60 brs	5.93 brs	5.89 s
8'	-	-	-	-	-
9'	2.28*	2.37 brs	2.26 brs	2.81 brs	2.66 brs
10'	-	-	-	-	-
11'	a 4.10 dd (9.80, 5.20) b 4.21 dd (9.80, 4.80)	a 4.04 dd (9.60, 6.00) b 4.20 dd (9.60, 3.30)	a 4.03 dd (9.60, 5.40) b 4.19 dd (9.60, 3.00)	a 4.18 dd (10.20, 5.40) b 4.29 dd (10.20, 3.00)	a 4.14 dd (9.60, 5.40) b 4.25 dd (9.60, 3.00)
12'	1.75 s	1.73 s	1.73 s	1.99 s	1.95 s
13'	1.17 s	1.02 s	1.05 s	1.23 s	1.17 s
14'	1.13 s	0.97 s	0.93 s	1.26 s	1.27 s
15'	1.18 s	0.96 s	0.95 s	1.07 s	1.02 s

*Overlapped with other signals

of the coumarin moiety, whereas the downfield signal at δ_C 216.4 was indicative of a ketone group belonging to the sesquiterpene unit. HSQC spectrum classified the carbon signals to four aliphatic methylenes at δ_C 38.8 (C-1'), 34.8 (C-2'), 24.3 (C-6'), and a pri-

mary alcoholic carbon at δ_C 67.0 characteristic for C-11' and nine methines, five of them for umbelliferone moiety at δ_C 113.4 (C-3), 143.7 (C-4), 129.2 (C-5), 113.6 (C-6), and 101.7 (C-8) and four for methyls at δ_C 25.6 (C-12'), 22.7 (C-13'), 25.6 (C-14'), and 14.9 (C-15'). The 1H NMR spectrum of **1** showed resonance characteristics of four methyl singlets at δ_H 1.75 (H-12'), 1.17 (H-13'), 1.13 (H-14'), and 1.18 (H-15') and three olefinic resonances at δ_H 6.28 (H-3), 7.66 (H-4), and 5.62 (H-7'). Three aromatic protons at δ_H 7.39 (H-5), 6.86 (H-6), and 6.85 (H-8) suggested the presence of a 7, 9, 10-trisubstituted benzene ring, which was supported by the ^{13}C NMR spectrum [Tables 1 and 2 and Figures 2 and 3].

Table 2. ^{13}C -NMR data obtained with compounds 1–5 (125.7 MHz, δ ppm)

Position of carbon	1	2	3	4	5
2	161.5	161.6	161.6	160.6	160.9
3	113.4	113.4	113.4	112.9	113.3
4	143.7	143.8	143.8	142.9	143.3
5	129.2	129.1	129.1	128.9	128.8
6	113.6	113.5	113.5	112.8	112.6
7	162.2	162.5	162.4	161.1	161.2
8	101.7	101.7	101.7	101.5	101.1
9	156.3	156.3	156.3	155.3	155.6
10	113.0	112.8	112.9	112.7	112.7
1'	38.8	32.2	38.2	31.1	37.3
2'	34.8	25.2	27.7	24.4	26.0
3'	216.4	76.2	79.2	76.1	78.5
4'	47.9	37.6	39.1	36.3	37.6
5'	51.5	43.8	49.7	57.1	62.0
6'	24.3	23.6	23.7	199.8	198.4
7'	124.0	124.2	124.1	129.6	129.5
8'	132.7	132.9	132.7	155.3	155.5
9'	53.4	53.9	54.2	54.8	54.7
10'	36.2	36.0	36.2	41.6	41.9
11'	67.0	67.5	67.4	65.9	65.3
12'	25.6	22.1	22.0	21.6	21.3
13'	22.7	28.5	28.4	21.4	14.8
14'	25.6	22.7	15.3	28.1	28.2
15'	14.9	15.2	15.2	16.1	15.5

The NMR spectral data of **2** were similar to those of **1**, except a slight difference in the signals assigned to the sesquiterpene unit which was due to the replacement of the ketone group at C-3' with an oxymethine. Regarding this portion and with respect to conferone, HMBC spectrum showed the correlations of the secondary alcoholic H-3' (δ_H 3.52) to δ_C 25.2 (C-2'), δ_C 37.6 (C-4'), and to δ_C 43.8 (C-5'). Other NMR spectral data of **2** were closely comparable to those of **1**. Hence, the structure of **2** was determined as mogoltacin (or conferol) [Tables 1 and 2 and Figures 4 and 5].

The NMR spectral data of **3** were very similar to those of **2**, except for the stereochemistry of C-3'. Thus, the differences between the 1H NMR of **2** and **3** appeared in the signals due to protons on C-2', C-3' and the methyl groups of 13' and 14'. In **3**, the signals for the H-2' and H-3' appeared at δ_H 1.67 and δ_H 3.31, respectively, and those for the methyl groups of 13' and 14' appeared at δ_H 1.05 and 0.93, respectively. In **2**, the corresponding signals appeared at δ_H 1.69 (H-2'), 3.52 (H-3'), 1.02 (H-13'), and 0.97 (H-14'), respectively. ROESY experiments confirmed the stereochemistry of 3'-OH as β and α conformers for compounds **2** and **3**, respectively. Therefore, compound **3** was determined as fesselol [Tables 1 and 2 and Figures 6 and 7].

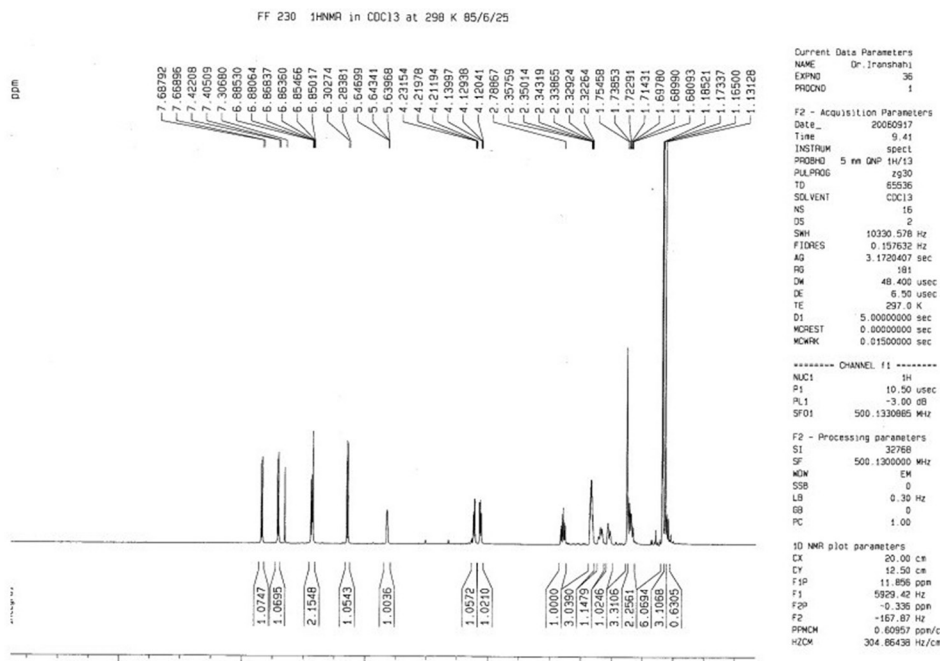


Figure 2. The 1H -NMR spectrum of conferone (**1**)

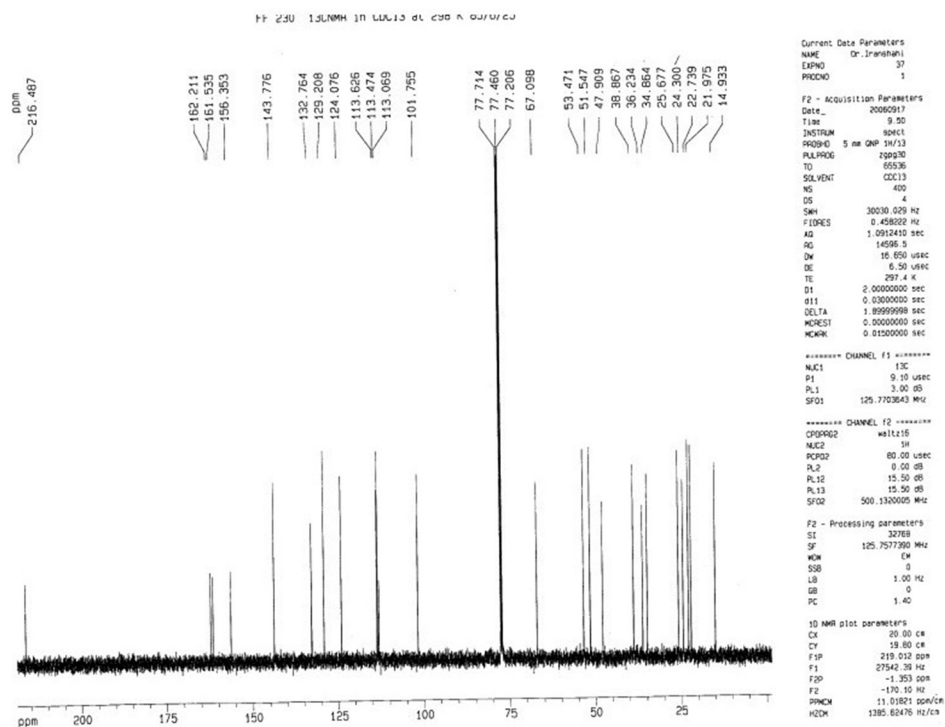


Figure 3. The ¹³C-NMR spectrum of conferone (1)

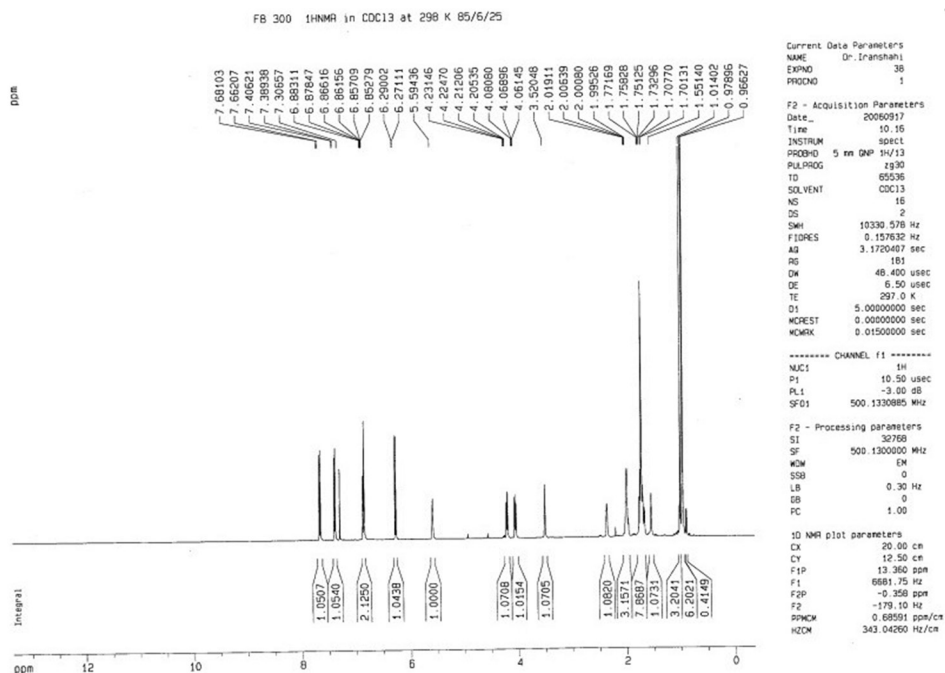


Figure 4. The ¹H-NMR spectrum of mogoltacin (2)

Based on a previous report on the P-gp inhibitory effect of diportlandin [4 in Figure 1], a sesquiterpene coumarin from *Euphorbia portlandica*,^[3] and conferone (1),^[4] we decided to test the activity of conferone, feselol, and mogoltacin on doxorubicin uptake in MCF-7/Dox cells. It should be pointed out that all of the tested compounds in this study and the previous one^[3] contain a

drimane-type sesquiterpene moiety linked to 7-hydroxycoumarin or 7-hydroxycoumarin derivatives.

Although breast cancer cells are usually sensitive to chemotherapy, P-gp overexpression and consequent resistance to chemotherapy is commonly observed in advanced breast tumors. Cells overexpressing P-gp are characterized by a lower capac-

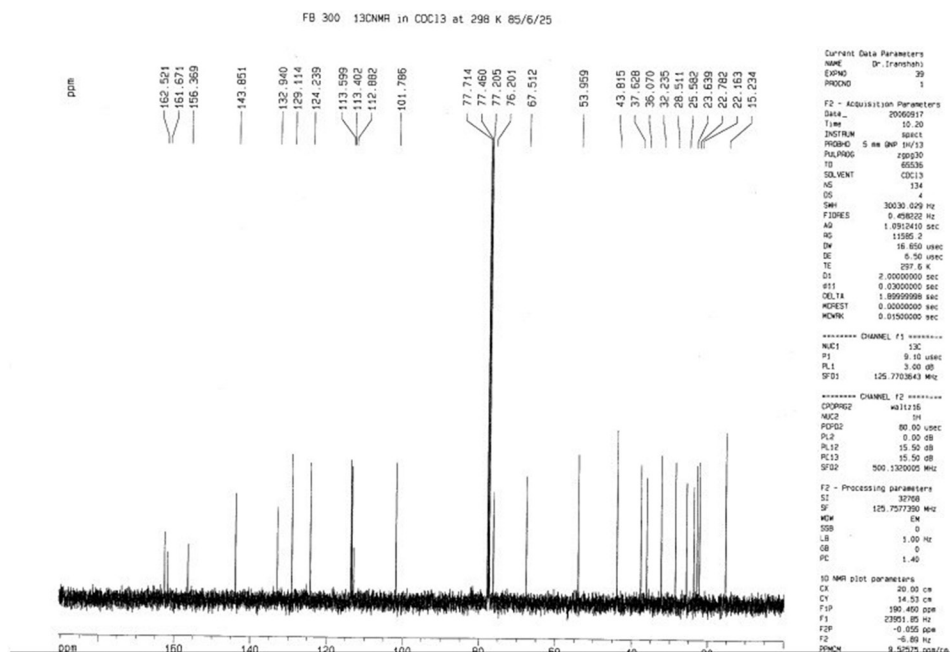


Figure 5. The ¹³C-NMR spectrum of mogoltacin (2)

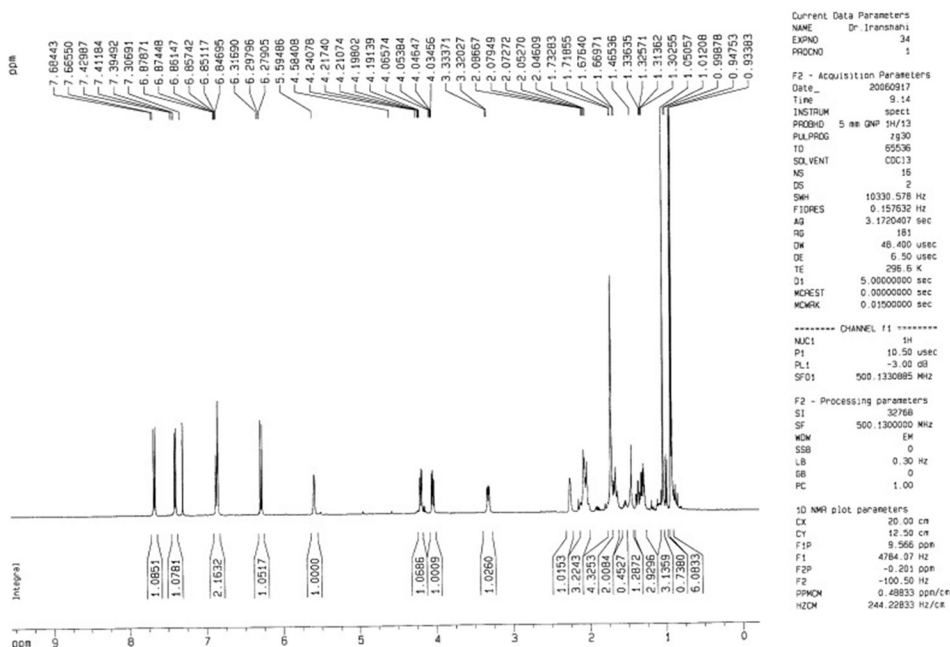


Figure 6. The ¹H-NMR spectrum of feselol (3)

ity to accumulate and retain anticancer drugs than the sensitive ones. Three hours after exposure to Dox, the MCF-7/Dox cells showed an intracellular concentration of Dox 12.1-fold lower than the parent sensitive MCF-7 cells. This marked difference in the intracellular Dox content demonstrates the overexpression of resistant human breast cancer cell line in drug efflux pumps. In a previous study, it was shown that diportlandin (4), a sesquiterpene coumarin with structural similarity to those of our tested compounds, has significant effect in inhibiting the

efflux pump activity mediated by P-gp, as compared to the positive control VPM.^[3] In 2006, Barthomeuf *et al.* reported that conferone (1) enhances the uptake of vinblastine in a mammalian cell line expressing the *MDR1* gene, evidencing for the first time that certain sesquiterpene ethers from *Ferula* may have some potency for the treatment of MDR malignancies.^[4] Furthermore, it was observed that the ability to inhibit P-gp transport is a common feature of various sesquiterpene coumarins from *Ferula* and that conferone may modulate *MDR1*

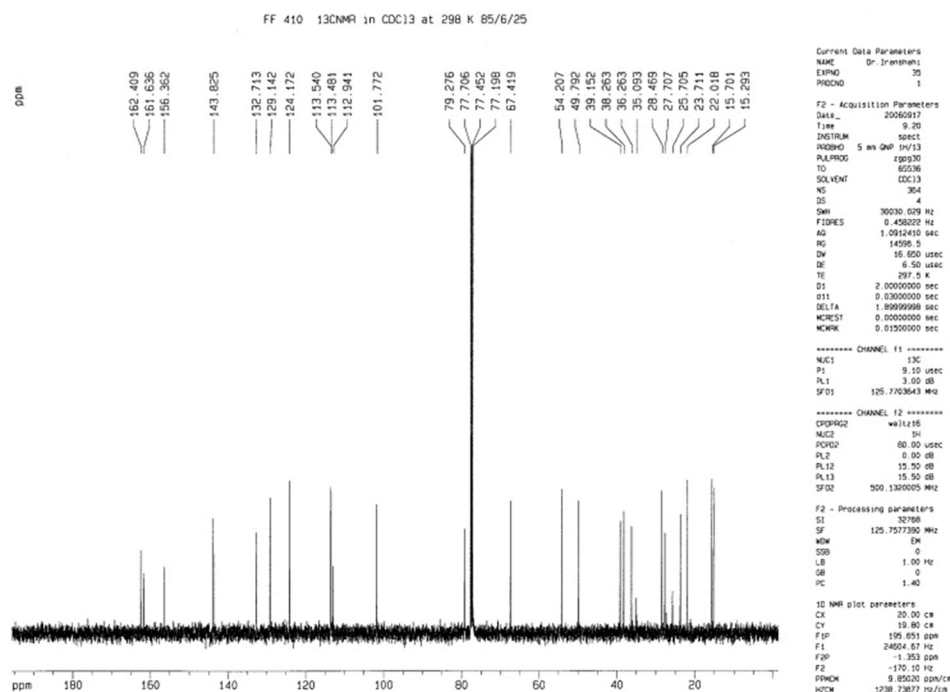


Figure 7. The ¹³C-NMR spectrum of feselol (3)

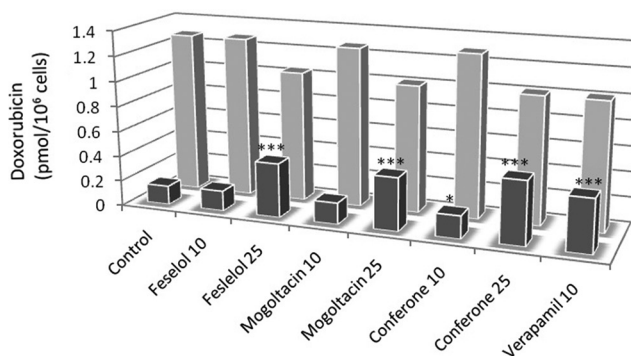


Figure 8. Enhanced accumulation of Dox in MCF-7/Dox cells by conferone, feselol, mogoltacin, and VPM. MCF-7/Dox (dark) and sensitive MCF-7 (light) cells were incubated with Dox (10 μ M) in the presence of the indicated concentrations of feselol, mogoltacin, and conferone. Control cells were not incubated with any compound. VPM (10 μ M) was used as positive control. Each experiment was performed in triplicate. P-values refer to the comparison between each tested compound and the negative control group. * $P < 0.05$; *** $P < 0.001$

gene expression.^[16] In the present study, it was verified that 3 h of exposure to feselol, mogoltacin, and conferone at 10 and 25 μ M, or to 10 μ M VPM singly, was not toxic for MCF-7 and MCF-7/Dox. Exposure of MCF-7/Dox to feselol, mogoltacin, conferone, or VPM enhanced Dox uptake [Figure 8]. This finding probably demonstrates that the tested coumarins have the capacity to inhibit P-gp-mediated drug efflux in human breast cancer cells. The chemosensitizing effects of coumarin derivatives were dose dependent, but varied with the structure of the chemical. At 25 μ M, all coumarins restored at least 50% of the reference uptake (uptake by sensitive cells); but at 10 μ M, their

potency varied in the order of conferone > mogoltacin > feselol. At this concentration, all compounds were less potent than the positive control. In sensitive MCF-7 cells, the uptake of Dox by cells was not affected by the addition of coumarins at 10 μ M concentration. However, it was decreased by VPM at 10 μ M and by the coumarins at 25 μ M. This decrease may be due to the enhanced cytotoxicity of the combination in sensitive MCF-7 cells, which caused less cell density and, therefore, less intracellular Dox to be assayed [Figure 2].

The present study probably proves that conferone, mogoltacin, and feselol suppress P-gp-mediated drug efflux in highly resistant human breast cancer cells. The most interesting finding is the possibility to establish the first structure-activity relationship, since all of the mentioned compounds have an identical carbon skeleton in their structures. On the other hand, previous findings^[4,9] demonstrate that drimane-type sesquiterpene coumarins have low toxicity and could serve as promising lead compounds for designing novel chemosensitizing agents. Although the toxicological profile of the tested phytochemicals is yet to be determined, it should be pointed out that *Ferula* species which contain these compounds have been traditionally used as medicinal plants, spices, and foods for a long time and, thus, could be considered as potentially safe.

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