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Antiproliferative and cytotoxic activities of furocoumarins of *Ducrosia anethifolia*

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**ABSTRACT**

**Context:** Phytochemical and pharmacological data on *Ducrosia anethifolia* (DC.) Boiss. (Apiaceae), an Iranian medicinal plant, are scarce; however, furocoumarins are characteristic compounds of *D. anethifolia*. 

**Objective:** Our experiments identify the secondary metabolites of *D. anethifolia* and assess their antitumor and anti-multidrug resistance activities.

**Materials and methods:** Pure compounds were isolated from the extract of aerial parts of the plant by chromatographic methods. Bioactivities were tested on multidrug resistant and sensitive mouse T-lymphoma cell lines. The inhibition of the cancer MDR efflux pump ABCB1 was evaluated by flow cytometry (at 2 and 20 μM). A checkerboard microplate method was applied to study the interactions of furocoumarins and doxorubicin. Toxicity was studied using normal murine NIH/3T3 fibroblasts.

**Results:** Thirteen pure compounds were isolated, nine furocoumarins namely, pabulenol, (+)-oxypeucedanin hydrate, oxypeucedanin, oxypeucedanin methanolate, imperatorin, isosopsoridol, heraclenin, heraclenol, and harmine along with stigmasterol and the furocoumarins (psoralen, isopsoralen) have been reported. Oxypeucedanin showed the highest activity against parent (IC50 ¼ 25.98 ± 1.27, 40.33 ± 0.63 μM) and multidrug resistant cells (IC50 ¼ 28.89 ± 0.73, 66.68 ± 0.00 μM), respectively, and exhibited slight toxicity on normal murine fibroblasts (IC50 ¼ 57.18 ± 3.91 μM).

**Discussion and conclusions:** Compounds 2, 3, 5, 7, 10–13 were identified for the first time from the *Ducrosia* genus. Here, we report a comprehensive *in vitro* assessment of the antitumor activities of *D. anethifolia* furocoumarins. Oxypeucedanin is a promising compound for further investigations for its anti-cancer effects.

**Introduction**

The genus *Ducrosia* (Apiaceae) consists of six species: *Ducrosia ismaelis* Asch., *D. flabellifolia* Boiss., *D. assadii* Alava., *D. areysiana* (Deflers) Pimenov & Kljuykov, *D. inaccessa* (C.C.Towns.) Pimenov & Kljuykov and *D. anethifolia* (DC.) Boiss. *D. anethifolia* is one of the three species growing wild in several areas of Iran, Afghanistan, Pakistan, Syria, Lebanon, Iraq, and some other Arab states and countries along the Persian Gulf (Aynehchi 1991; Ghahtreman 1993; Mozaffarian 1996). The whole herb, especially its aerial part, has been used in Iranian folk medicine as an analgesic and in case of anxiety and insomnia (Shalaby et al. 2014). The aerial part, including the seed was reported to be carminative and useful for irregularities of menstruation and galactagogue (Amiri and Joharchi 2016). The herb is added to a variety of Persian foods for flavouring (Aynehchi 1991; Haghhi et al. 2004). The phytochemical profile of *D. anethifolia* has only been partly explored. In the literature, the majority of papers deal with the composition of the essential oil (EO). As major constituents, α-pinene (11.6% (Mostafavi et al. 2008), 70.3% (Mottaghipisheh et al. 2014), 59.2% (Janssen et al. 1984)); n-decanal (1.4-45% (Karami and Bohlooeli 2017), 45.06% (Vazirzadeh et al. 2017), 70% (Hajhashemi et al. 2010), 57% (Maboubi and Feizabadi 2009), 25.6–30.3% (Mazloomifar and Valian 2015), 18.8% (Sefidkon and Javidtash 2002)), dodecanal (28.8% (Shahabipour et al. 2013)), cis-chrysanthene (72.28%) (Ashraf et al. 1979; Hajhashemi et al. 2010), 57% (Maboubi and Feizabadi 2009), 25.6–30.3% (Mazloomifar and Valian 2015), 18.8% (Sefidkon and Javidtash 2002)), dodecanal (28.8% (Shahabipour et al. 2013)), cis-chrysanthene (72.28%) (Ashraf et al. 1979; Hajhashemi et al. 2010), 57% (Maboubi and Feizabadi 2009), 25.6–30.3% (Mazloomifar and Valian 2015), 18.8% (Sefidkon and Javidtash 2002)),
The bioactivities of the extracts of aerial parts of D. anethifolia have been studied in vitro and in vivo. Different extracts of the plant exerted moderate anti-radical scavenging (Mottaghipisheh et al. 2014; Shahat et al. 2015); and antibacterial effects (Syed et al. 1987; Mahboubi and Feizabadi 2009). Pangelin isolated from D. anethifolia demonstrated activity against a panel of fast growing mycobacteria (Stavri et al. 2003). Essential oil of the seeds and methanol extract showed a weak antibacterial effect against 14 Gram positive and negative bacteria (Javidnia et al. 2009; Habibi et al. 2017). In an experiment on three human cancer cell lines (K562, LS180 and MCF-7), D. anethifolia EO demonstrated remarkable to moderate cytotoxic activity, while EO of D. flabellifolia showed less pronounced activity (Shahabipour et al. 2013). Ducchisin B exerted remarkable cytotoxicity against the human colon HCT-116 and ovary SKOV-3 cancer cell lines in vitro (Queslati et al. 2017).

The crude D. anethifolia extract and the isolated furocoumarins exhibited in vivo antidiabetic activities (Shalaby et al. 2014). The in vivo anxiolytic (Hajlashemi et al. 2010; Shokri et al. 2013; Zamyad et al. 2016), sedative (Hajlashemi et al. 2010), analgesic and anti-inflammatory (Asagari Nemati et al. 2017) and also anti-locomotor activities (Zamyad et al. 2016) of D. anethifolia EO have been tested. Intra-peritoneal administration of the D. anethifolia EO improved spatial learning and memory in adult male rats (Abbasnejad et al. 2017). The intra-peritoneal injection of the hydroalcoholic extract of D. anethifolia effectively reduced the pentyleneetetrazole-induced seizure manifestations in male Wistar rats (Nyasty et al. 2017). Moreover, D. anethifolia extract reduced the number of germ cells, the level of testosterone and spermatogenesis in male Wistar rats (Rahimi et al. 2013; Zamyad et al. 2016), sedative (Hajhashemi et al. 2010), anti-locomotor activity (Shahabipour et al. 2013). Ducrosin B exerted remarkable cytotoxic effect against a panel of fast growing mycobacteria (Stavri et al. 2003).

As presented above, furocoumarins are the most characteristic compounds of the Ducrosia and their activities against cancer cells seem to be promising. Imperatorin showed antiproliferative effect on human hepatoma HepG2 cells (Luo et al. 2011); furthermore, this compound and heracalenin induced apoptosis in Jurkat leukemia cells. In Jurkat cells treated for 72h with heracalenin and imperatorin, most of the DNA fragmentation occurred at the G2/M and G1/S phases of the cell cycle, respectively (Appendino et al. 2004). 8-Methoxypsoralen inhibited the growth of neuroblastoma (IC50 = 56.3 µM) and metastatic colon cancer cells (IC50 = 88.5 µM) by triggering both extrinsic and intrinsic apoptotic pathways, independently of photoactivation (Bartnik et al. 2017). Isoimperatorin, cnidicin, imperatorin, oxy- peucedanin, byakangelicol and oxypeucedanin hydrate exhibited a significant inhibition on cell proliferation in a dose-dependent manner, particularly oxypeucedanin against HCT-15 (colon cancer) cells with ED50 = 3.4 ± 0.3 µg/mL (Kim et al. 2007).

Beside direct antiproliferative and cytotoxic activities, furocoumarins affect multidrug resistance (MDR) as well. Among 20 selected furocoumarin derivatives, phellopterin (IC50 = 8.0 ± 4.0 µM) and iso- pimpinellin (IC50 = 260 ± 5.7 µM) exhibited the highest activity against CEM/C1 (lymphoblastic leukemia) and HL-60/MX2 (MDR) cell lines, respectively (Kubrak et al. 2017). Feroninell A reverted MDR in A549RT-eto lung cancer cells (Kaewpiboon et al. 2014). Bergapten (IC50 = 40.29 ± 0.30 nM) and xanthotoxin (IC50 = 1.10 ± 0.91 nM) showed remarkable anticancer activity against EPG85. 257RDB (MDR1 overexpressing human gastric adenocarcinoma cell line) and MCF7MX (BCRP overexpressing human epithelial breast cancer cell line), respectively (Mirzai et al. 2017).

Our work explores the phytochemical composition of D. anethifolia, examines the complex in vitro anticancer activities, including antiproliferative, cytotoxic and anti-MDR effects of its isolated compounds, and analyses the interaction of compounds possessing promising bioactivities with chemotherapeutics.

**Materials and methods**

**General procedures**

NMR spectra were recorded in CD3OD and CDCl3 on a Bruker Avance DRX 500 spectrometer at 500 MHz (1H) and 125 MHz (13C). The peaks of the residual solvent (δ1H 3.31 and 7.26, δ13C 49.0 and 77.2, respectively) were taken as reference. The data were acquired and processed with MestReNova v6.0.2e-5475 software. Chemical shifts are expressed in parts per million and coupling constants (J) values are reported in Hz. All solvents were used in analytical grade (Molar Chemicals Kft, Halásztelek, Hungary).

Pure compounds were isolated by using open column chromatography (Silica gel 60, 0.063–0.2 mm, Merck, Darmstadt, Germany) (CC), medium pressure liquid chromatography (MPLC, silica gel 60, 0.045–0.063 mm, Merck, Darmstadt, Germany), gel chromatography (Sephadex® LH-20, Pharmacia, Uppsala, Sweden), normal (Silica gel 60 RP-18 F254S, Merck, Darmstadt, Germany) and reverse phase (Silica gel 60 RP-18 F254S, Merck, Darmstadt, Germany) preparative thin layer chromatography (PTLC and RP-PTLC, respectively), centrifugal PTLC (Silica gel 60 GF254, Merck, Darmstadt, Germany) (CPTLC) and reverse phase preparative HPLC (Kinetex® 5 µm C-18 100 Å, 150 x 4.6 mm Phenomenex, Torrance, CA) (RP-HPLC). The HPLC flow was 1.2 mL/min, column oven temperature was 24 °C. Detection was carried out within the range of 190–800 nm. The HPLC system comprised of Waters 600 pump, Waters 2998 PDA detector, Waters in/line degasser AF degasser unit connected with Waters 600 control module using Empower Pro 5.0 software.

**Plant material**

The aerial parts of Ducrosia anethifolia were collected by JM from south of Iran (Fars, Neyriz, Iran) in April 2016. Identification of the plant was done by Dr. Mohammad Jamal Saharkhiz at Department of Horticultural Science, Faculty of Agriculture, Shiraz University, Iran, and a voucher specimen was deposited in the Herbarium of Department of Pharmacoognosy, University of Szeged (voucher no.: 880).

**Isolation of compounds**

Aerial parts (flower, leaves and stem, 3 kg) were dried in shade at room temperature and powdered, then extracted with methanol (40 L). After filtration, the filtrate was concentrated under reduced pressure to yield the crude extract. The extract (464.1 g) was dissolved with methanol–water 1:1 (1.5 L) and then partitioned successively with n-hexane (4 x 1 L), CHCl3 (4 x 1 L), EtOAc (4 x 1 L) and n-BuOH (4 x 1 L). The solvents were removed from each extract to yield the n-hexane extract, CHCl3 extract, EtOAc extract and n-BuOH extract.

The CHCl3-soluble fraction (20.6 g) was initially subjected to CC with a gradient system consisting of increasing concentration of MeOH in CHCl3 (0–100%); column fractions with similar TLC patterns were combined to get six major fractions D1-D6. Fractions D1-D6 were chromatographed by MPLC, first eluting with n-hexane–CH2Cl2 (50:50; 0:100), then adding MeOH to CH2Cl2 (0–100%), to afford four subfractions (D11, D12, D13 and D14). D14 was separated to 49 subfractions using CPTLC with anocratic eluting system n-hexane–EtOAc–MeOH (10:3:1), which
resulted in the isolation of the pure compound 6 (82.8 mg). The RP-HPLC purification of D11 subfractions with MeOH–H2O (MeOH–H2O 1:1) afforded compound 10 (1.7 mg). D12 was chromatographed by MPLC applying a gradient solvent system with increasing EtOAc in n-hexane (5–100%) to get eight major subfractions (D121–D128). From D123, the pure compound 3 (3.1 mg) was isolated by using CPTLC with EtOAc in n-hexane (5–100%). D124 was successively separated to 81 fractions by CPTLC (same system), then subfractions 49–54 was subjected to RP-HPLC with MeOH–H2O (15–50% H2O in MeOH) yielding compound 9 (2.56 mg).

D13 was separated by MPLC with increasing ratio of EtOAc in n-hexane (5–100%) to get seven fractions (D131–D137). D133 was subjected to MPLC with the same solvent system to gain 19 subfractions. Finally, subfractions 1–2 were purified by using CPTLC with toluene–EtOAc (90:10, 80:20, 70:30, 60:40, 50:50) as eluents to gain compound 2 (100.4 mg). Subtraction 3 from D133 was subjected to CPTLC by eluting with cyclohexane–EtOAc (90:10, 80:20, 70:30, 60:40, 50:50) to yield 32 subfractions. Subfractions 18–19 of D133 were separated by PTLC with toluene–EtOAc (1:1) to get compound 8 (35.3 mg). Besides, subfractions 23–32 were chromatographed by RP-HPLC (MeOH–H2O 1:1) and then by PTLC with toluene–EtOAc (1:1) to yield compounds 12 (1.02 mg) and 1 (1.78 mg). By using CPTLC with increasing concentration of EtOAc in toluene (5–100%) as eluents, subfraction 6 from D133 was chromatographed to get 43 subfractions. Subfractions 24–28 and 36–40 were separated by PTLC with CHCl3–MeOH–n-hexane (5:1:5) to retrieve compounds 4 (2.5 mg) and 7 (21.7 mg), respectively.

D3 was separated to five major fractions (D31–D35) by MPLC with a solvent system containing increasing ratio of MeOH in CHCl3 (0–100%). D33 was chromatographed by CPTLC with raising the concentration of MeOH (0–20%) in the mixture of cyclohexane–EtOAc (1:1) to afford 70 subfractions. Subfractions 21–23 contained the pure compound 11 (1.0 mg). The main fraction D4 was separated by MPLC to seven subfractions (D41–D47) by raising the ratio of MeOH (0–100%) in acetone–toluene (1:1). Subfraction D42 was subsequently chromatographed by PTLC with eluting cyclohexane–EtOAc–MeOH (4.75:4.75:0.5) and compound 5 (2.7 mg) was isolated. Furthermore, D46 was purified with CPTLC by increasing ratio of MeOH (0–100%) in acetone–toluene (1:1); then compound 13 (2.9 mg) was purified by using RP-PTLC [MeOH–H2O (1:1)] from subfractions 35–37 (Figure 1).

**Cell lines**

L5178Y mouse T-cell lymphoma cells: parent, PAR cells (ECACC cat. no. 87111908, obtained from FDA, Silver Spring, MD) were transfected with pHa MDRI/A retrovirus. The ABCB1-expressing L5178Y cell line (MDR) was selected by culturing the infected cells in 60 ng/mL colchicine containing medium. L5178Y PAR mouse T-cell lymphoma cells and the L5178Y human ABCB1-transfected subline (MDR) were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, 200 mM l-glutamine, and penicillin–streptomycin mixture in 100 U/L and 10 mg/L concentration, respectively, at 37°C and in a 5% CO2 atmosphere.

NIH/3T3 mouse embryonic fibroblast cell line (ATCC CRL-1658) was purchased from LGC Promochem (Teddington, UK). The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO), containing 4.5 g/L glucose, supplemented with 10% heat-inactivated foetal bovine serum (FBS). The cells were incubated at 37°C, in a 5% CO2, 95% air atmosphere.

**Assay for antiproliferative effect**

The effects of increasing concentrations of the analysed compounds on cell proliferation were tested in 96-well flat-bottomed microtiter plates (Poljarević et al. 2018). The compounds were diluted in 100 μL of McCoy's 5A medium. 6 × 104 mouse T-cell lymphoma cells (PAR or MDR) in medium (100 μL) were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37°C for 72 h; at the end of the incubation period, 20 μL of MTT solution
(thiazolyl blue tetrazolium bromide, Sigma, St. Louis, MO) (from a 5 mg/mL stock) was added to each well. After incubation at 37°C for 4 h, 100 μL of sodium dodecyl sulphate (SDS, Sigma, St. Louis, MO) solution (10% in 0.01 M HCl) was added to each well and the plates were further incubated at 37°C overnight. The cell growth was determined by measuring the OD at 540 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Waltham, MA). IC50 values were calculated via the following equation:

\[
\text{IC}_{50} = 100 - \left( \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{medium control}}}{\text{OD}_{\text{cell control}} - \text{OD}_{\text{medium control}}} \right) \times 100
\]

**Assay for cytotoxic effect**

The effects of increasing concentrations of compounds on cell growth were tested in 96-well flat-bottomed microtiter plates (Poljarvec et al. 2018). The compounds were diluted in a volume of 100 μL medium. Then, 1 × 10⁴ cells in 100 μL of medium were added to each well, with the exception of the medium control wells. In case of NIH/3T3 cells, the compounds were added after seeding the cells at 37°C overnight. The culture plates were incubated at 37°C for 24 h; at the end of the incubation period, 20 μL of MTT solution (from a 5 mg/mL stock) was added to each well. After incubation at 37°C for 4 h, 100 μL of SDS solution (10% in 0.01 M HCl) was added to each well and the plates were further incubated at 37°C overnight. Cell growth was determined by measuring the optical density (OD) at 540 nm (ref. 630 nm) with a Multiscan EX ELISA reader. Inhibition of the cell growth was determined according to the formula:

\[
\text{IC}_{50} = 100 - \left( \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{medium control}}}{\text{OD}_{\text{cell control}} - \text{OD}_{\text{medium control}}} \right) \times 100
\]

Results are expressed in terms of IC50, defined as the inhibitory dose that reduces by a 50% the growth of the cells exposed to the tested compound.

**Assay for multidrug resistance reversing activity**

The inhibition of the cancer MDR efflux pump ABCB1 by the tested compounds was evaluated using flow cytometry measuring the retention of rhodamine 123 by ABCB1 (P-glycoprotein) in MDR mouse T-lymphoma cells, as the L5178Y human ABCB1 gene transfected mouse T-lymphoma cell line (MDR) overexpresses P-glycoprotein (Domínguez-Álvarez et al. 2016). This method is a fluorescence-based detection system which uses verapamil as reference inhibitor. Briefly, cell number of L5178Y MDR and PAR cell lines was adjusted to 2 × 10⁴ cells/mL, re-suspended in serum-free McCoy’s 5A medium and distributed into 0.5 mL aliquots into Eppendorf centrifuge tubes. The tested compounds were added at different concentrations and the samples were incubated for 10 min at room temperature. Verapamil (Sigma, St. Louis, MO) and tarividular (Sigma, St. Louis, MO) were applied as positive controls. Next, 10 μL (5.2 μM final concentration) of the fluorochrome and ABCB1 substrate rhodamine 123 (Sigma, St. Louis, MO) were added to the samples and the cells were incubated for 20 min at 37°C, washed twice and re-suspended in 0.5 mL PBS for analysis. The fluorescence of the cell population was measured with a Partec CyFlow® flow cytometer (Partec, Görlitz, Germany). The percentage of mean fluorescence intensity was calculated for the treated MDR cells as compared with the untreated cells. A fluorescence activity ratio (FAR) was calculated based on the following equation which relates the measured fluorescence values:

\[
\text{FAR} = \frac{\text{MDR}_{\text{treated}}}{\text{MDR}_{\text{control}}} = \frac{\text{MDR}_{\text{parental treated}}}{\text{MDR}_{\text{parental control}}}
\]

The results obtained from a representative flow cytometry experiment in which 20,000 individual cells of the population were evaluated for amount of rhodamine 123 retained with the aid of the Partec CyFlow® flow cytometer, are first presented by the histograms and these data converted to FAR units that define fluorescence intensity, standard deviation, peak channel in the total- and in the gated-populations. Parameters calculated are: forward scatter (FSC, forward scatter count of cells in the samples or cell size ratio); side scatter (SSC, side scatter count of cells in the samples); FL1 (mean fluorescence intensity of the cells) and FAR, whose values were calculated using the equation given above.

**Checkerboard combination assay**

A checkerboard microplate method was applied to study the effect of drug interactions between furocoumarins and the chemotherapeutic drug doxorubicin (Takács et al. 2015). This assay was carried out using multidrug resistant mouse T-lymphoma cells overexpressing the ABCB1 transporter. Doxorubicin is in the class of anthracycline antitumor agents, and it exerts anticancer activity as a topoisomerase-II (TI-2) inhibitor. The dilutions of doxorubicin (Teva, Debrecen, Hungary, stock solution: 2 mg/mL) were made in a horizontal direction in 100 μL (final concentration: 17.242 μM), and the dilutions of the test compounds vertically in the microtiter plate in 50 μL volume. The cells were re-suspended in McCoy’s 5A culture medium and distributed into each well in 50 μL containing 6 × 10⁴ cells each. The plates were incubated for 72 h at 37°C in 5% CO₂ atmosphere. The cell growth rate was determined after MTT staining. At the end of the incubation period, 20 μL of MTT solution (from a stock solution of 5 mg/mL) was added to each well. After incubation at 37°C for 4 h, 100 μL of SDS solution (10% in 0.01 M HCl) was added to each well and the plates were further incubated at 37°C overnight. Optical density was measured at 540/630 nm with Multiscan EX ELISA reader (Thermo Labsystems, Waltham, MA) as described elsewhere (Takács et al. 2015). Combination index (CI) values at 50% of the growth inhibition dose (ED50) were determined using CompuSyn software (ComboSyn, Inc., Paramus, NJ) to plot four to five data points to each ratio. CI values were calculated by means of the median-effect equation, where CI < 1, CI = 1 and CI > 1 represent synergism, additive effect (or no interaction) and antagonism, respectively (Chou and Martin 2005; Chou 2010).

**Results**

**Isolated compounds**

Repeated column chromatography of the bioactive fractions resulted in the isolation of 13 compounds. The compounds were identified by careful interpretation of NMR data and comparison of ¹H and ¹³C chemical shifts with those reported in literature. Nine linear furocoumarin derivatives, namely pabulene (1) (Sbai et al. 2016), (+)-oxypeucedanin hydrate (aviprin) (2) (Sbai et al. 2016), oxypeucedanin (3) (Sbai et al. 2016), oxypeucedanin methanolate (4) (Fujioka et al. 1999), (-)-oxypeucedanin hydrate (prangol) (5) (Rahimifard et al. 2018), imperatorin (6) (Lv et al.
Table 1. Antiproliferative (AA) and cytotoxic activities (CA) of the furocoumarins against PAR, MDR and NIH/3T3 cells presented as IC50 values.

| Compounds                   | AA on PAR cells (μM) | AA on MDR cells (μM) | CA on PAR cells (μM) | CA on MDR cells (μM) | CA on NIH/3T3 cells (μM) |
|-----------------------------|----------------------|----------------------|----------------------|----------------------|--------------------------|
| Pabulenol (1)               | 30.47 ± 0.47         | 29.28 ± 0.45         | 51.32 ± 3.32         | >100                 | 54.09 ± 3.83             |
| (+)-Oxypeucedanin hydrate (2)| 41.96 ± 0.88         | 60.58 ± 2.74         | >100                 | >100                 | 83.55 ± 0.57             |
| Oxypeucedanin (3)           | 25.98 ± 1.27         | 28.89 ± 0.73         | 40.33 ± 0.63         | 66.68 ± 0.00         | 57.18 ± 3.91             |
| Oxypeucedanin methanolate (4)| 35.88 ± 0.96         | 33.23 ± 0.51         | 56.42 ± 2.53         | >100                 | 47.16 ± 1.28             |
| Imperatorin (6)             | 36.12 ± 0.91         | 42.24 ± 0.88         | 52.56 ± 4.19         | >100                 | 92.41 ± 2.80             |
| Isogosospherol (7)          | 46.53 ± 0.47         | 48.75 ± 0.28         | >100                 | >100                 | 54.82 ± 1.19             |
| Heraclenin (8)              | 32.73 ± 2.40         | 46.54 ± 1.22         | 65.81 ± 1.00         | 83.94 ± 1.68         | 70.91 ± 4.26             |
| Heraclenol (9)              | 52.31 ± 2.12         | 46.57 ± 0.47         | >100                 | >100                 | 65.78 ± 0.46             |
| Doxorubicin                 | 0.054 ± 0.005        | 0.468 ± 0.065        | 0.377 ± 0.02         | 7.152 ± 0.358        | 5.71 ± 0.50              |

Data were expressed as mean ± standard deviation (n = 3). Different letters represent significant differences (p < 0.05).

Table 2. Efflux pump inhibiting activities of furocoumarins.

| Samples                  | Conc. μM | FSC | SSC | FL-1 | FAR |
|--------------------------|----------|-----|-----|------|-----|
| PAR                      | 2069     | 658 | 98.2| 2     | –   |
| MDR                      | 2152     | 725 | 1.79| –    |     |
| MDR mean                 |          |     | 1.182|     |
| Tariquidar               | 0.02     | 2156| 719 | 119  | 100.68 |
| Verapamil                | 2143     | 740 | 9.69 | 8.2  |
| Pabulenol (1)            | 2        | 2324| 728 | 0.596| 0.82 |
| (+)-Oxypeucedanin hydrate(2)| 2       | 2323| 750 | 0.544| 0.75 |
| Oxypeucedanin (3)        | 2        | 2190| 499 | 0.76 | 
| Oxypeucedanin methanolate(4)| 2       | 2164| 763 | 2.62 | 2.22 |
| Imperatorin (6)          | 2        | 2165| 749 | 0.727| 0.62 |
| Isogosospherol (7)       | 2        | 2305| 741 | 0.531| 0.73 |
| Heraclenin (8)           | 2        | 2318| 723 | 0.942| 1.29 |
| Heraclenol (9)           | 2        | 2184| 764 | 0.573| 0.78 |
| DMSO                     | 2% (V/V) | 2308| 762 | 0.497| 0.68 |
| MDR                      | –        | 2301| 746 | 0.535|     |

Table 3. Checkerboard combination assay of selected compounds with doxorubicin.

| Compound                  | Best ratio | CI at ED50 | Interaction | SD (±) |
|---------------------------|------------|------------|-------------|-------|
| Oxypeucedanin             | 1:50       | 0.8553     | Slight synergism | 0.078000 |
| Heraclenin                | 4:100      | 0.88955    | Slight synergism | 0.063344 |

Furocoumarins isolated from *D. anethifolia* were subjected to bioassay for cytotoxic and antiproliferative activity against cancer cell lines. All compounds exerted potent antiproliferative effect on sensitive and resistant mouse T-lymphoma cells (Table 1). However, they did not show any selectivity towards the resistant cell line. The most potent compound was oxypeucedanin on both cell lines. Some compounds had no toxic effects (+)-oxypeucedanin hydrate (2), heraclenol (9), isogosospherol (7); furthermore, pabulenol (1), oxypeucedanin methanolate (4) and imperatorin (6) were more toxic on the sensitive PAR cell line (IC50 between 52 and 57 μM) without any toxicity on MDR cells (Table 1). Oxypeucedanin (3) and heraclenin (8) exhibited cytotoxic activity; however, they were more potent on the sensitive PAR cell line (Table 1). The cytotoxic activity of furocoumarins was assessed using NIH/3T3 normal murine fibroblast cells. Some compounds showed slight toxic effect on normal fibroblasts, namely (+)-oxypeucedanin hydrate (2), heraclenol (4) and isogosospherol (8) with IC50 values of 83.55, 65.78 and 54.82 μM, respectively. Pabulenol (1) possessed similar activity on fibroblast and parental mouse lymphoma cells. In addition, oxypeucedanin (3), oxypeucedin methanolate (5) and heraclenin (9) exhibited mild toxicity on fibroblasts and parental lymphoma cells. Imperatorin (7) had no toxic activity on fibroblasts.

### Multidrug resistance reversing activity

Regarding the efflux pump inhibiting activity of the compounds on ABCB1 overexpressing MDR mouse T-lymphoma cells, only oxypeucedanin (3) showed moderate ABCB1 inhibiting effect (FAR: 2.22); however, this inhibition was lower than in case of the positive controls tariquidar (FAR: 100) and verapamil (FAR: 8.2) (Table 2, figures see in Supporting Information).

### Combination assay results on MDR cells

The two most promising compounds in the previous assays were investigated in combination with the standard chemotherapeutic drug doxorubicin. The compounds oxypeucedanin (3) and heraclenin (8) showed slight synergistic effect with doxorubicin, for this reason, they might be potential adjuvants in combined chemotherapy applying standard anticancer drugs with compounds that can act synergistically (Table 3).
Discussion

Chromatographic separation of the extract of *D. anethifolia* herbs resulted in the isolation of 13 compounds, among them were nine furocoumarins. Compounds 2, 3, 5, 7, 10–13 were identified for the first time from *Ducrosia* genus.

The tested furocoumarins exerted antiproliferative effects on sensitive and resistant mouse T-lymphoma cells with no selectivity towards the resistant cell line. This is the first comprehensive analysis of this plant and its furocoumarins on these cells. Oxypeucedanin (3) had the most remarkable activity on both cell lines. The most effective furocoumarins, oxypeucedanin (3) and heraclenin (9) exhibited marginal toxicity on normal fibroblast cells and sensitive parental mouse lymphoma cells; furthermore, they were less toxic on multidrug resistant lymphoma cells. From the tested compounds, only oxypeucedanin showed moderate MDR reversing activity. In the checkerboard assay, oxypeucedanin and heraclenin showed slight synergistic effect with doxorubicin. These compounds might improve the cytotoxic effect of the standard chemotherapeutic drug doxorubicin.

Disclosure statement

The authors declare no conflict of interest.

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