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RESEARCH ARTICLE

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 (**1**), (+)-oxypeucedanin hydrate (**2**), oxypeucedanin (**3**), oxypeucedanin methanolate (**4**), (−)-oxypeucedanin hydrate (**5**), imperatorin (**6**), isogospherol (**7**), heraclenin (**8**), heraclenol (**9**), along with vanillic aldehyde (**10**), harmine (**11**), 3-hydroxy-α-ionone (**12**) and 2-*C*-methyl-erythrytol (**13**). Oxypeucedanin showed the highest *in vitro* antiproliferative and cytotoxic activity against parent (IC$_{50}$ = 25.98 ± 1.27, 40.33 ± 0.63 μM) and multidrug resistant cells (IC$_{50}$ = 28.89 ± 0.73, 66.68 ± 0.00 μM), respectively, and exhibited slight toxicity on normal murine fibroblasts (IC$_{50}$ = 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 anticancer 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; Ghahreman 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; Haghi 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 Bohlooli 2017), 45.06%

(Vazirzadeh et al. 2017), 70% (Hajhashemi et al. 2010), 57% (Mahboubi 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*-chrysanthenyl acetate (72.28%) (Ashraf et al. 1979; Habibi et al. 2017) have been reported.

Furocoumarins and terpenoids are characteristic components of the *Ducrosia* genus. From the seeds of *D. anethifolia*, two new terpenoids, the monoterpene ducrosin A and the sesquiterpene ducrosin B were isolated along with stigmasterol and the furocoumarins heraclenin and heraclenol (Queslati et al. 2017). Psoralen, 5-methoxypsoralen, 8-methoxypsoralen, imperatorin, isooxypeucedanin, pabulenol, pangelin, oxypeucedanin methanolate, oxypeucedanin hydrate, 3-*O*-glucopyranosyl-β-sitosterol and 8-*O*-debenzoylpaeoniflorin were also isolated from the extract of *D. anethifolia* (Stavri et al. 2003; Shalaby et al. 2014). GC analysis of the fatty acids showed high percentages of elaidic acid and oleic acid (Queslati et al. 2017), beside 58.8% petroselinic acid in the seed oil of *D. anethifolia* (Khalid et al. 2009). Apart from *D. anethifolia*, furocoumarins (psoralen, isopsoralen) have been reported only from *D. ismaelis* from this genus (Morgan et al. 2015).

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📄 Supplemental data for this article can be accessed here.

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). Ducrosin 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 (Hajhashemi et al. 2010; Shokri et al. 2013; Zamyad et al. 2016), sedative (Hajhashemi et al. 2010), analgesic and anti-inflammatory (Asgari Nematian 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 pentylenetetrazole-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. 2016).

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 heraclenin induced apoptosis in Jurkat leukemia cells. In Jurkat cells treated for 72 h with heraclenin 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 ($IC_{50} = 56.3$ μM) and metastatic colon cancer cells ($IC_{50} = 88.5$ μM) by triggering both extrinsic and intrinsic apoptotic pathways, independently of photoactivation (Bartnik et al. 2017). Isoimperatorin, cnidicin, imperatorin, oxypeucedanin, 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 $ED_{50} = 3.4 \pm 0.3$ 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 ($IC_{50} = 8.0 \pm 4.0$ μM) and isopimpinellin ($IC_{50} = 26.0 \pm 5.7$ μM) exhibited the highest activity against CEM/C1 (lymphoblastic leukaemia) and HL-60/MX2 (MDR) cell lines, respectively (Kubrak et al. 2017). Feroniellin A reverted MDR in A549RT-eto lung cancer cells (Kaewpiboon et al. 2014). Bergapten ($IC_{50} = 40.29 \pm 0.30$ nM) and xanthotoxin ($IC_{50} = 1.10 \pm 0.91$ 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 (Mirzaei 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 CD_3OD and $CDCl_3$ on a Bruker Avance DRX 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C). The peaks of the residual solvent (δ_H 3.31 and 7.26, δ_C 49.0 and 77.2, respectively) were taken as reference. The data were acquired and processed with MestReNova *v*6.0.2e-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, Merck, Darmstadt, Germany) and reverse phase (Silica gel 60 RP-18 F$_{254}$s, Merck, Darmstadt, Germany) preparative thin layer chromatography (PTLC and RP-PTLC, respectively), centrifugal PTLC (Silica gel 60 GF$_{254}$, Merck, Darmstadt, Germany) (CPTLC) and reverse phase preparative HPLC (Kinetex® 5 μm C-18 100 Å, 150 × 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 5.00 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 Pharmacognosy, 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 × 1 L), CHCl$_3$ (4 × 1 L), EtOAc (4 × 1 L) and *n*-BuOH (4 × 1 L). The solvents were removed from each extract to yield the *n*-hexane extract, CHCl$_3$ extract, EtOAc extract and *n*-BuOH extract.

The CHCl$_3$-soluble fraction (20.6 g) was initially subjected to CC with a gradient system consisting of increasing concentration of MeOH in CHCl$_3$ (0–80%); column fractions with similar TLC patterns were combined to get six major fractions D$_1$, D$_2$, D$_3$, D$_4$, D$_5$ and D$_6$. D$_1$ was chromatographed by MPLC, first eluting with *n*-hexane–CH$_2$Cl$_2$ (50:50; 0:100), then adding MeOH to CH$_2$Cl$_2$ (0–100%), to afford four subfractions (D$_{11}$, D$_{12}$, D$_{13}$ and D$_{14}$). D$_{11}$ was separated to 49 subfractions using CPTLC with an isocratic eluting system *n*-hexane–EtOAc–MeOH (10:3:1), which
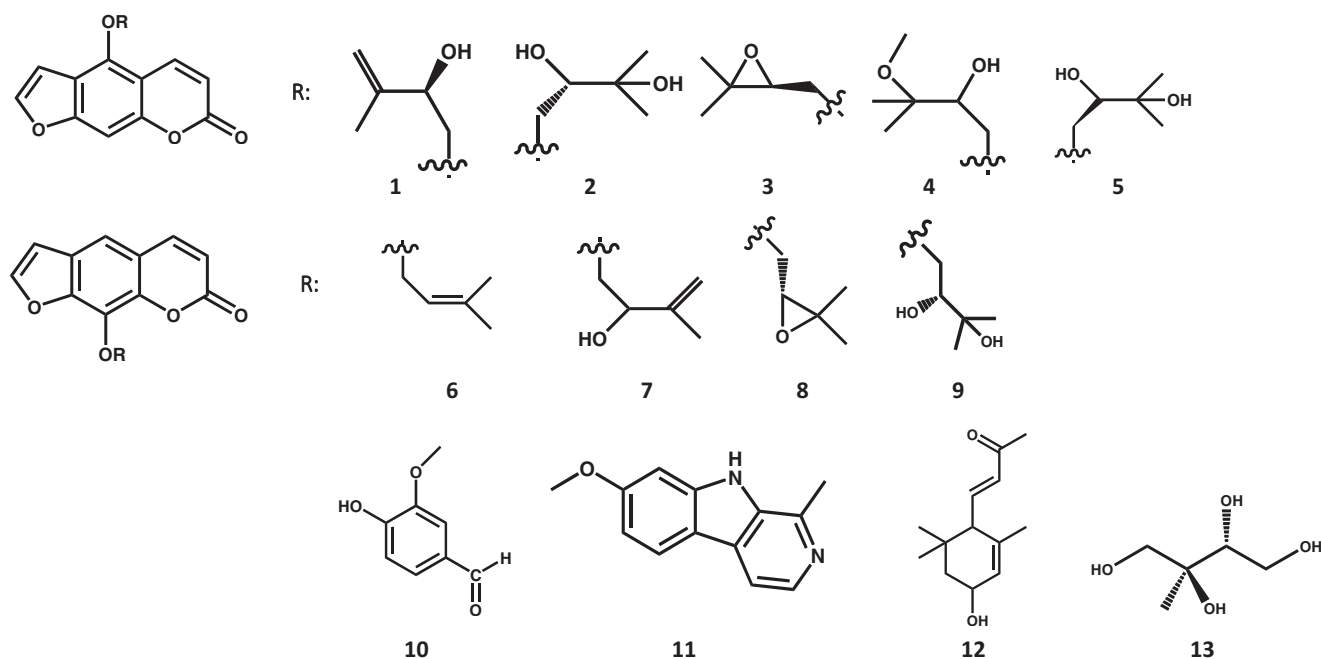
Figure 1. Secondary metabolites isolated from *Ducrosia anethifolia*.

resulted in the isolation of the pure compound **6** (82.8 mg). The RP-HPLC purification of D_{11} subfractions with MeOH–H_2O (MeOH–H_2O 1:1) afforded compound **10** (1.7 mg). D_{12} was chromatographed by MPLC applying a gradient solvent system with increasing EtOAc in *n*-hexane (5-100%) to get eight major subfractions (D_{121}–D_{128}). From D_{123}, the pure compound **3** (3.1 mg) was isolated by using CPTLC with EtOAc in *n*-hexane (5–100%). D_{124} was successively separated to 81 fractions by CPTLC (same system), then subfractions 49–54 was subjected to RP-HPLC with MeOH–H_2O (15–50% H_2O in MeOH) yielding compound **9** (2.56 mg).

D_{13} was separated by MPLC with increasing ratio of EtOAc in *n*-hexane (5–100%) to get seven fractions (D_{131}–D_{137}). D_{133} 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). Subfraction 3 from D_{133} was subjected to CPTLC by eluting with toluene–EtOAc (90:10, 80:20, 70:30, 60:40, 50:50) to yield 32 subfractions. Subfractions 18–19 of D_{133} 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–H_2O 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 D_{133} was chromatographed to get 43 subfractions. Subfractions 24–28 and 36–40 were separated by PTLC with $CHCl_3$–MeOH–*n*-hexane (5:1:5) to retrieve compounds **4** (2.5 mg) and **7** (21.7 mg), respectively.

D_3 was separated to five major fractions (D_{31}–D_{35}) by MPLC with a solvent system containing increasing ratio of MeOH in $CHCl_3$ (0–100%). D_{33} 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 D_4 was separated by MPLC to seven subfractions (D_{41}–D_{47}) by raising the ratio of MeOH (0–100%) in acetone–toluene (1:1). Subfraction D_{42} 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, D_{46} 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–H_2O (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 MDR1/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% CO_2 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% CO_2, 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. 6×10^3 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). IC_{50} values were calculated via the following equation:

$$IC_{50} = 100 - \left[\frac{OD_{sample} - OD_{mediumcontrol}}{OD_{cellcontrol} - OD_{mediumcontrol}} \right] \times 100$$

Assay for cytotoxic effect

The effects of increasing concentrations of compounds on cell growth were tested in 96-well flat-bottomed microtiter plates (Poljarević et al. 2018). The compounds were diluted in a volume of 100 μL medium. Then, 1×10^4 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:

$$IC_{50} = 100 - \left[\frac{OD_{sample} - OD_{mediumcontrol}}{OD_{cellcontrol} - OD_{mediumcontrol}} \right] \times 100$$

Results are expressed in terms of IC_{50}, 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 2×10^6 cells/mL, re-suspended in serum-free McCoy's 5A medium and distributed in 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 tariquidar (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:

$$FAR = \frac{MDR_{treated}/MDR_{control}}{parental_{treated}/parental_{control}}$$

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); FL-1 (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 6×10^3 cells each. The plates were incubated for 72 h at 37 °C in 5% CO_2 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 (ED_{50}) 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 ^{13}C chemical shifts with those reported in literature. Nine linear furocoumarin derivatives, namely pabulenol (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 IC_{50} 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/3T3cells (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 ± 5.23	>100	47.16 ± 1.28
Imperatorin (**6**)	36.12 ± 0.91	42.24 ± 0.88	52.56 ± 4.19	>100	92.41 ± 2.80
Isogospherol (**7**)	46.53 ± 0.47	48.75 ± 0.28	>100	>100	54.82 ± 1.99
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	98.20	–
MDR	–	2152	725	1.79	–
			MDR mean	1.182	–
Tariquidar	0.02	2156	719	119	100.68
Verapamil	20	2143	740	9.69	8.20
Pabulenol (**1**)	2	2324	728	0.596	0.82
	20	2323	750	0.544	0.75
(+)-Oxypeucedanin hydrate (**2**)	2	2325	725	0.715	0.98
	20	2305	769	0.583	0.80
Oxypeucedanin (**3**)	2	2190	749	0.9	0.76
	20	2164	763	2.62	2.22
Oxypeucedanin methanolate (**4**)	2	2310	737	0.58	0.80
	20	2290	766	0.499	0.68
Imperatorin (**6**)	2	2165	749	0.727	0.62
	20	2161	750	0.783	0.66
Isogospherol (**7**)	2	2305	741	0.531	0.73
	20	2326	741	0.548	0.75
Heraclenin (**8**)	2	2318	723	0.942	1.29
	20	2294	764	0.57	0.78
Heraclenol (**9**)	2	2300	742	0.995	1.37
	20	2317	740	0.348	1.30
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 ED_{50}	Interaction	SD (±)
Oxypeucedanin	1:50	0.85537	Slight synergism	0.07800
Heraclenin	4:100	0.88955	Slight synergism	0.06334

2013), isogospherol (**7**) (Macias et al. 1990), heraclenin (**8**) (Poonkodi 2016), heraclenol (**9**) (Harkar et al. 1984); along with vanillic aldehyde (**10**) (Chung et al. 2011), harmine (**11**), 3-hydroxy-α-ionone (**12**) and 2-C-methyl-erythrytol (**13**) were identified (^{1}H NMR spectra see in Supporting Information). The diastereomers (+)-oxypeucedanin hydrate and (−)-oxypeucedanin hydrate were distinguished by determining their optical rotations and comparing with literature (Atkinson et al. 1974).

The ^{1}H and ^{13}C NMR spectral data of **11**, **12** and **13** in CD_3OD are reported here for the first time. Compound **11** (harmine): ^{1}H NMR (500 MHz, CD_3OD) $\delta = 8.11$ (H-3, d, $J = 5.5$ Hz), 8.02 (H-5, d, $J = 8.7$ Hz), 7.86 (H-4, d, $J = 5.5$ Hz), 7.06 (H-8, d, $J = 1.9$ Hz), 6.89 (H-6, dd, $J = 8.7$ Hz, 1.9 Hz), 3.92 (s, 7-OCH$_3$), 2.80 (s, 1-CH$_3$); ^{13}C NMR (125 MHz, CD_3OD) $\delta = 162.9$, 144.6, 141.6, 137.0, 136.2, 130.7, 123.7, 116.3, 113.5, 111.4, 95.4, 56.0, 19.1. Compound **12** (3-hydroxy-α-ionone): ^{1}H NMR (500 MHz, CD_3OD) $\delta = 6.67$ (H-7, dd, $J = 15.8$ Hz, 10.3 Hz), 6.13 (H-8, d, $J = 15.8$ Hz), 5.60 (H-4, br s), 4.22 (H-3, br s), 2.58 (H-6, d, $J = 10.3$ Hz), 2.27 (H$_3$-10, s), 1.80 (H-2β, dd, $J = 13.2$ Hz, 5.9 Hz), 1.63 (H$_3$-13, s), 1.38 (H-2α, dd, $J = 13.2$ Hz, 7.2 Hz), 1.01 (H$_3$-11, s), 0.90 (H$_3$-12, s); ^{13}C NMR (125 MHz, CD_3OD) $\delta = 200.8$, 149.8, 135.9, 134.7, 127.3, 65.9, 55.6, 45.0, 35.0, 29.8, 27.1, 24.5, 22.8. Compound **13** (2-C-methyl-erythrytol): ^{1}H NMR (500 MHz, CD_3OD) $\delta = 3.80$ (H-4a, dd, $J = 10.4$ Hz, 2.5 Hz), 3.61 (H-3, m), 3.59 (H-4b, m), 3.52 (H-1a, d, $J = 11.1$ Hz), 3.44 (H-1b, d, $J = 11.1$ Hz), 1.11 (2-CH$_3$, s); ^{13}C NMR (125 MHz, CD_3OD) $\delta = 76.2$, 75.0, 68.5, 63.8, 19.7.

Antiproliferative and cytotoxic activities on cancer cell lines

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**), isogospherol (**7**)); furthermore, pabulenol (**1**), oxypeucedanin methanolate (**4**) and imperatorin (**6**) were more toxic on the sensitive PAR cell line (IC_{50} 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 isogospherol (**8**) with IC_{50} 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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