**Daucane esters from laserwort (*Laserpitium latifolium* L.) inhibit cytokine and chemokine production in human lung epithelial cells**

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

*Background:* Laserwort, *Laserpitium latifolium* L. (Apiaceae), is a European medicinal plant. Its roots and rhizomes were traditionally used as a general tonic and to treat inflammatory and infective diseases.

*Purpose:* The anti-inflammatory potential of daucane esters, isolated from underground parts extract of *L. latifolium* and specific structural features that contribute to their activity were investigated. In addition, we studied their interference with the transactivation capacity of the Glucocorticoid Receptor when added together with a classic glucocorticoid (GC), dexamethasone (DEX). This particular property may be relevant in combination strategies, attempting to circumvent diabetogenic side effects of glucocorticoids upon long-term anti-inflammatory treatments.

*Materials and methods:* Nine *L. latifolium* daucane esters were isolated and elucidated as derivatives of desoxodehydrolaserpitin, laserpitin and a novel 2β-esterified laserpitinol analogue. Of all compounds effects on NF-κB- and AP-1-driven pro-inflammatory pathways were assessed using TNF- or PMA-induced reporter gene analysis in A549 cells. Daucanes with a strong and concentration-dependent inhibition of both NF-κB and AP-1, were tested for a potential effect on DEX-stimulated GR-driven Glucocorticoid Response Element (GRE) reporter gene activity. In addition, GRE-driven anti-inflammatory mRNA expression was determined (GILZ and DUSP1). Also anti-inflammatory properties were validated by monitoring effects on CCL-2, IL-6, IL-1β mRNA expression levels (qPCR) and on CCL-2 chemokine production (ELISA).

*Results:* Daucanes featuring an ester moiety and/or a hydroxy group at positions 2β, 6α and 10α and especially the novel 2β-esterified laserpitinol derivative that, in comparison to other isolated compounds, features an additional 9α-hydroxy group, demonstrated suppression of both NF-κB- and AP-1-dependent pro-inflammatory pathways. Remarkably, those entities competitively and concentration-dependently repressed GR-driven GRE-dependent reporter gene activities. The most active compounds inhibited CCL-2 protein excretion and compound **4** downregulated genes coding for IL-1β and IL-6 induced upon TNF treatment in A549. In absence of TNF, compound **4** upregulated the GRE-mediated anti-inflammatory gene GILZ, but not DUSP1.

*Conclusions:* Daucane esters are novel anti-inflammatory agents that may, in combination with GCs, potentially improve therapeutic benefit. These results contribute to the ongoing search for novel anti-inflammatory agents as safer alternatives to, or with, GCs.

*Keywords:*

Anti-inflammatory activity, Glucocorticoid Receptor,NF-κB, AP-1, Chemokine production, Inducible gene expression

*Abbreviations:*

GC, glucocorticoid; DEX, dexamethasone; NF-κB, nuclear factor-κB; AP-1, activator protein 1; TNF, tumor necrosis factor; PMA, phorbol-12-myristate-13-acetate; GRE, glucocorticoid response element; CCL-2, chemokine (C-C motif) ligand 2; IL, interleukin; qPCR, quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay;PEPCK, phosphoenolpyruvate carboxykinase; DUSP1, dual specificity protein phosphatase 1; GILZ, glucocorticoid-induced leucine zipper; CHCl3, chloroform; HR-TOF-MS, high-resolution time-of-flight mass spectrometry; ESI, electrospray ionization; NMR, nuclear magnetic resonance; 2-D NMR two-dimensional NMR spectroscopy; CDCl3, deuterochloroform; Me, methyl; 1HNMR, proton NMR; 13CNMR, carbon NMR; DMEM, Dulbecco's modified eagle medium; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; 36B4, 60S acidic ribosomal protein P0; UBE2D2, ubiquitin conjugating enzyme E2 D2; HMBC, heteronuclear multiple bond correlation; NOESY, nuclear Overhauser effect spectroscopy; NO, nitric oxide; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthases; COX-2, cyclooxygenase-2.

**Introduction**

An inflammatory reaction is a systemic immune response of cells and tissues to various harmful stimuli, such as bacterial lipopolysaccharides, tumor necrosis factor (TNFα), irradiation or viral infection (Beck et al., 2015). It represents a protection of the body and ceases once the harmful agents have been removed. Chronic inflammation, by contrast, is the continued presence (sometimes over many years) of pro-inflammatory factors at levels higher than baseline. Therefore, it is associated with many age-related diseases, including diabetes, atherosclerosis, Alzheimer’s disease, osteoarthritis and cancer (Freund et al., 2010). Pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and phorbol esters (PMA), trigger a signaling cascade that activates two main transcription factors in cells, NF-κB and AP-1, respectively. These transcription factors regulate expression of genes coding for cytokines, chemokines, inflammatory enzymes and receptors, as well as adhesion molecules that support the migration of immune cells towards inflammatory sites in the body (Rothe et al., 1995; Van Bogaert et al., 2011). A most effective treatment for many inflammatory diseases includes administration of synthetic glucocorticoids (GCs), such as e. g. prednisolone or dexamethasone. Glucocorticoids are steroidal ligands for the Glucocorticoid Receptor (GR). Among other mechanisms, activated GR can mediate transrepression of NF-κB- and AP-1- pro-inflammatory pathways, hence contributing to an anti-inflammatory activity. Yet, GCs represent a double-edged sword upon chronic usage, with beneficial therapeutic effects contrasting to severe adverse effects on glucose regulation. Diabetogenic effects arise from the activation of glucocorticoid response element (GRE)-dependent gene expression, by triggering key rate-limiting enzymes involved in gluconeogenesis, e.g. PEPCK and Glucose-6-phosphatase (Sundahl et al., 2015).This side effect typical becomes an issue upon long-term treatment regimens. It overshadows the inflammation-resolving effect of a classic GC treatment in which GRE-driven anti-inflammatory genes (e.g. DUSP1, GILZ) are desirable to efficiently help controlling a short-term and more acute inflammatory insult (Clark and Belvisi, 2012; De Bosscher et al., 2016). The glucocorticoid receptor exhibits a range of different functionalities, and therefore the development of safer ligands that trigger only desirable subsets of genes is challenging (De Bosscher, 2010). Yet, recent investigations revealed a number of novel, non-steroidal selective GR modulators that favor GR transrepression, hereby stimulating anti-inflammatory properties, while not affecting GR transactivation, hereby avoiding typical side effects, such as e.g. hyperglycemia (Sundahl et al., 2015).

Laserwort, *Laserpitium latifolium* L. (Apiaceae) is a European medicinal species of which roots and rhizomes have been used in Russian and German traditional medicine to alleviate symptoms connected to inflammation and infection (Vereskovskii et al., 1992; Hegi 1906). Analyses of the chemical composition revealed that roots and rhizomes of this species were particularly rich in daucane esters (Holub et al., 1967a, 1967b; Moldt et al., 1987, Popović et al., 2013). In previous investigations, cytotoxic and antimicrobial properties of the extract of the underground parts and laserpitin were tested (Popović et al., 2013; Popović et al., 2015), but no data on its anti-inflammatory activity were reported so far.

In the current study, in search for novel and safer glucocorticoid alternatives, the anti-inflammatory potential of daucane constituents of *L. latifolium* was investigated. Compared to classic GCs, we wanted to test if daucane esters exhibit a so-called dissociative profile, i.e. if they can repress NF-κB and AP-1 as central transcription factors in inflammation, not only without stimulating GRE-driven gene expression, which are associated with metabolic side effects of glucocorticoids (De Bosscher et al., 2015), but also with the capacity to inhibit GC-driven GRE gene expression. Molecules that emerged to be potent inhibitors of NF-κB and AP-1inhibited also GR-mediated and GRE-driven gene expression. Notably, the most active daucane exhibited a peculiar target gene-specific profile by stimulating mRNA levels of the GRE anti-inflammatory gene GILZ, but not DUSP1. In line with the anti-inflammatory action on reporter genes, the active daucanes also suppressed mRNA expression and CCL-2 production, as a typical inflammation marker in TNF-induced A549 cells.

**Material and methods**

*Plant material and extract preparation*

The plant material (roots and rhizomes of *L. latifolium*) was collected at Mt. Gučevo, Serbia in October 2008. It was identified by Dr. Marjan Niketić from the Natural History Museum in Belgrade. The voucher specimen is deposited at the same institute under accession number ko03102008. The underground parts of *L. latifolium* were extracted and re-extracted with CHCl3 as reported previously (Popović et al., 2013).The full procedure on extraction and chromatography conditions and compound isolation is given as Supplementary material.

*Chemicals and reagents*

The solvents were of the highest quality commercially available and purchased from Sigma Aldrich (Diegem, Belgium) and Biosolve (Valkenswaard, The Netherlands). Dexamethasone (DEX) and Phorbol-12-Myristate-13-Acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant mouse TNF, used in the NF-κB reporter assay, was produced in *E. coli* and purified to homogeneity at The Protein Production Core Facility PSF, VIB Ghent University. TNF had a specific activity of 1.2 × 108 IU/mg and had no detectable endotoxin contamination. The preparation of the luciferase assay reagent used was reported previously (De Bosscher et al., 2014).

*Structural elucidation of compounds*

Laserpitin, diangeloyloxydauc-8-ene-4β-ol (**1**), paliin (**2**), desoxodehydrolaserpitin (**3**), 6α-deangeloyloxy-6α-isobutyriloxy-laserpitine (**5**) and acetyldesoxodehydrolaserpitin (**8**) were previously isolated and their structure was elucidated based on comparison of obtained with reported spectral data. Laserol monoangeloyloxy (**6**) was isolated as a transparent solid substance(CHCl3), and by 2-D NMR it was concluded that it is a novel isomer of a monoangeloyloxy-laserol (esterified at position 6α instead of 10α).1H NMR (500 MHz, CDCl3): *δ*H 0.75 (3H, d 7.0 Hz, Me-13), 0.87 (3H, d 7.0 Hz, Me-12), 1.24 (1H, m, overlapped, H-2β), 1.29 (3H, s, Me-14), 1.45 (3H, s, Me-15), 1.55 (1H, m, overlapped, H-3β), 1.60 (1H, m, overlapped, H-11), 1.63 (1H, m, overlapped, H-2α), 1.99 (1H, dd 14.0, 10.0 Hz, H-3α), 1.97 (1H, d 11.0 Hz, H-5), 2.28 (1H, dd 17.0, 1.5 Hz, H-7α), 2.40 (1H, ddd 17.0, 5.0, 1.5 Hz, H-7β), 4.43 (1H, d 8.5 Hz, H-10), 5.48 (1H, ddd 11.0, 5.0, 1.5 Hz, H-6); *Angeloyloxy* : *δ*H 1.87 (3H, m), 2.03 (3H, m), 6.05 (1H, qq 7.3, 1.5 Hz).13C NMR (125 MHz, CDCl3): *δ*C 48.5 (C-1), 33.8 (C-2), 30.6 (C-3), 84.8 (C-4), 51.3 (C-5), 69.1 (C-6), 39.5 (C-7), 75.5 (C-8), 211.0 (C-9), 79.8 (C-10), 36.5 (C-11), 17.5 (C-12), 17.2 (C-13), 28.5 (C-14), 21.5 (C-15); *Angeloyloxy*: *δ*C 168.5, 140.1, 128.0, 20.1, 15.3 (**Fig.** **S4** and **S5**, Supplementary material). HR-TOF-MS (ESI negative) *m/z* [M-H]- 367.2134, calcd. 367.2126 for C20H31O6) (**Fig. S6**, Supplementary material).

10α-Acetoxy-2β,6α-diangeloyloxy-dauca-4β,8α,9α-triol (**4**) was isolated as a transparent colourless solid substance (CHCl3), 1H NMR (500 MHz, CDCl3): *δ*H 0.90 (3H, d 6.8 Hz, Me-13); 0.94 (3H, d 6.8 Hz, Me-12), 1.36 (3H, s, Me-14), 1.46 (3H, s, Me-15), 1.57 (1H, m, H-3α), 1.80 (1H, m, H-11), 1.90 (1H, m, H-7α), 2.05 (1H, m, H-7β), 2.47 (1H, d 11.0 Hz, H-5), 2.48 (1H, dd 13.5, 9 Hz, H-3β), 2.94 (1H, d 5.5 Hz, H-9), 4.66 (1H, dd 10.0, 9.0 Hz, H-2), 4.88 (1H, d 5.5 Hz, H-10), 5.39 (1H, td 11.0, 1.8 Hz, H-6); *Acetyloxy*: *δ*H2.11 (3H, s); *Angeloyloxy 1*: *δ*H1.88 (3H, m), 1.99 (3H, m), 6.12 (1H, qq 7.3, 1.5 Hz); *Angeloyloxy 2*:*δ*H1.91 (3H, m), 2.00 (3H, m), 6.14 (1H, qq 7.4, 1.5 Hz). 13C NMR (125 MHz, CDCl3): *δ*C 48.6 (C-1), 74.2 (C-2), 36.5 (C-3), 81.6, (C-4), 48.8 (C-5), 68.4 (C-6), 42.5 (C-7), 55.9 (C-8), 59.1 (C-9), 69.0 (C-10), 36.8 (C-11), 18.2 (C-12), 17.0 (C-13), 12.9 (C-14), 23.2 (C-15); *Acetyloxy*: *δ*C20.5, 169.6; *Angeloyloxy 1*: *δ*C167.5, 139.1, 127.3, 20.7, 16.0; *Angeloyloxy 2*:*δ*C167.8, 140.1, 127.5, 20.7, 16.0 (**Fig.** **S1** and **S2**, Supplementary material). HR-TOF-MS (ESI positive) *m/z* [M-2H2O+H]+ 475.2710, calcd. 457.2707 for C27H39O7 (**Fig. S3**, Supplementary material).

10α-Deangeloyloxy-10α-(2,3-dyhydroxyl-2-methyl-butanoiloxy)-laserpitin (**7**) was isolated as a transparent liquid (CDCl3), 1HNMR (500 MHz, CDCl3) (**Fig. S7**, Supplementary material): *δ*H 0.76 (3H, d 7.0 Hz, Me-12); 0.89 (3H, d 7.0 Hz, Me-13), 1.39 (3H, s, Me-15), 1.44 (3H, s, Me-14), 1.55 (1H, m, H-2α), 1.60 (1H, m, H-3α), 1.63(1H, m, H-11), 1.69 (1H, m, 2β-H), 2.00 (1H, m, 3β-H), 2.07 (1H, d 11.3 Hz, H-5), 2.35 (1H, dd 17.0, 1.0 Hz, H-7α), 2.48 (1H, dd 17.0, 5.2, H-7β), 5.27 (1H, s, H-10), 5.52 ddd (1H, ddd 11.3, 5.0, 1.2 Hz, H-6); *Angeloyloxy*: *δ*H1.87 (3H, m), 2.03 (3H, m), 6.16 (1H, qd 7.3, 1.5 Hz); *2,3-dihydroxy-2-methylbutanoyloxy*:*δ*H 1.25 (3H, d 6.8 Hz), 1.38 (3H, s), 4.13 (1H, q 6.8 Hz). 13CNMR (125 MHz, CDCl3): *δ*C 45.9 (C-1), 34.0 (C-2), 31.5 (C-3), 84.5 (C-4), 51.5 (C-5), 68.0 (C-6), 40.0 (C-7), 76.5 (C-8), 209.5 (C-9), 82.4 (C-10), 36.0 (C-11), 17.0 (C-12), 18.0 (C-13), 21.8 (C-14), 28.5 (C-15); *Angeloyloxy*: *δ*C168.0; 141.5; 128.0; 20.5; 15.5; *2,3-dihydroxy-2-methylbutanoyloxy*: *δ*C175.2; 78.0; 72.1; 20.8; 15.0. Transparent liquid (CHCl3), HR-TOF-MS (ESI positive) *m/z* [M+Na]+ 507.2568, calcd. 507.2564 for C25H40NaO9 (**Fig. S10**, Supplementary material).

*Cell cultures*

The human lung epithelial cell line A549 was obtained from ATCC (cell bank) and stably transfected with specific reporter genes, as indicated in the Figure legends, using a lentiviral transduction method (TronoLab, Lausanne, Switzerland). Basal A549 cells were stably transfected with pMet-NeoLuc, a plasmid expressing constitutively both Neomycine and luciferase, and then put on a selection for 3 weeks with geneticin (1 mg/ml)to obtain so-called “NeoLuc” cells used for cell viability tests. Cells were cultivated in DMEM (Gibco-Invitrogen, Merelbeke, Belgium) enriched with 10% fetal calf serum (International Medical Products, Brussels, Belgium), and supplemented with antibiotics: 100 IU/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cell cultures were maintained at 37 °C in a 5% CO2 atmosphere with 95% humidity. Subconfluent cells (80%) were passaged, using a solution of Gibco® Trypsin-EDTA (Gibco-Invitrogen, Merelbeke, Belgium).

*Reporter gene assays*

The effect of isolated daucanes on a TNF-induced stably integrated recombinant NF-κB reporter gene activity in A549 cells, on PMA-induced stably integrated recombinant AP-1 reporter gene activity and on constitutive Neo-Luc activity in A549 cells was determined using a previously reported methodology (De Bosscher et al., 2005). Briefly, 9000 cells per well were seeded and incubated for 24h in a 96-well plate. After incubation, a test compound or Dexamethasone or a solvent control were added to the wells. After 1h, an induction using TNFα or PMA was performed in cells with stably integrated NF-κB-or AP-1-dependent reporter genes, respectively. Hereafter, plates were incubated for 5h and following the cell lysis using PBS buffer, luciferase assays were carried out by a protocol reported by Promega Corp. (Madison, WI, USA) and measured using TopCount luminometer (Packard Instrument Company, Meriden, Connecticut, USA). The compounds were tested at three different concentrations (60, 30 and 10 μM) to enable observation of specific, concentration-dependent gene repression. NF-κB- and AP-1-dependent reporter gene activities of cells that were treated only with TNF or PMA in presence of solvent controls were put at 100%. To calculate an IC50 value, the most active daucane **4** was tested under the same conditions at five different concentrations in the range of 1-60 μM. Cell viability tests on NeoLuc A549 cells were conducted in parallel for the compounds applied at three concentrations (60, 30 and 10 μM) using the same protocol as for reporter gene assays. For assays monitoring the activity of the Glucocorticoid Response Element (GRE)-dependent promoter fragment coupled to luciferase, also a stably integrated reporter gene construct in A549 cells was generated using the lentiviral transduction method (Tronolab, Lausanne).

*Enzyme-linked Immunosorbent Assay (ELISA)*

For ELISA experiments, an eBioscience Human CCL-2 (MCP-1) ELISA Ready-Set-Go!® kit was used (San Diego, CA, USA), according to the manufacturer’s instructions. A549 cells were seeded in a 6-well plate (250.000 cells/well). The second day, a 24-hour starvation was performed in wells using Opti-MEM (Gibco-Invitrogen, Merelbeke, Belgium). Hereafter, compounds (30 μM) or solvent control or DEX (1 μM) were added to wells, followed by TNF (200 IU/ml) after one hour. Cells were next incubated for 3 h after which the medium was collected and either used directly, or else kept at – 80°C prior to analyses.

*mRNA purification and qPCR analyses*

Total RNA was purified using the RNeasy+ kit (Qiagen, Venlo, Nederlands). After removal of medium, 1% of β-Mercaptoethanol was added to the wells and mRNA was extracted according to a protocol given by the producer (Qiagen, Venlo, Nederlands). RNA was transcribed to cDNA with the PrimeScriptTM 1st strand cDNA Synthesis Kit (Takara Bio Inc.). RT-qPCR analysis was performed using the Lightcycler 480 system and Lightcycler qPCR 480 SYBRGreen I master reagents (Roche Diagnostics, Vilvoorde, Belgium), to assay specific human CCL-2, IL-1β, IL-6, GILZ, DUSP1, 36B4, UBE2D2 and cyclophilin A mRNA levels. qPCR was performed in triplicate. All primer sequences are available upon request. The signal was normalized to reference genes as a control, using Genorm-Q Base (Biogazelle).

*Statistical and numerical analyses*

The results shown in Tables 1-3 and Figures 2-5 are presented as the mean values ± standard error of the mean (SEM) of at least three independent replicates. Statistical significances between the test and control groups (significance levels \*p≤0.05; \*\*p≤0.01; \*\*\*p≤0.001) were determined by One-way ANOVA tests followed by a Dunett’s test (GraphPad Prism4).

**Results and discussion**

*Isolation and structure elucidation of daucanes*

From the methanol-soluble fraction of the chloroform extract of the underground parts of *L. latifolium* after detailed optimisation of the separation conditions used before (Popović et al., 2013), nine daucane esters were isolated and their structure elucidated (**Fig. 1**). Our analysis revealed the presence of laserpitin (Popović et al., 2013, Holub et al., 1967a), acetyldesoxodehydrolaserpitin (**8**) (Popović et al., 2013), desoxodehydrolaserpitin (**3**) (Holub et al., 1967a) and 6α-deangeloyloxy-6α-isobutyriloxy-laserpitine (**5**) (Moldt et al.,1987) as known constituents of extracts of roots and rhizomes of *L. latifolium.* We identified five additional daucane esters that were not isolated from this species before.2β,6α-Diangeloyloxydauc-8-ene-3β-ol (**1**) was previously isolated from the rhizomes of *Ferula jaeschkeana* Vatke (Garg et al. 1990). 6α,10α-Diangeloyloxydauc-8-ene-3β-ol (**2**) also known as paliin or 6α,10α-diangeloyl-jaeschkeanadiol, was previously isolated from roots of *Ferula glauca* L. (Dall’Acqua et al., 2011), *F. pallida* Korov., and *F. latipinna* A. Santos (Kushmuradov et al., 1986). In the obtained NMR spectra for compound **4**, some of the characteristic patterns resembled to those of a laserpitinol, i.e. daucan-4β,8α,9α-triol featuring angeloyl ester moieties at C-6 and C-10 (Holub et al., 1970). However, unlike laserpitinol, **4** featured a α-oriented acetic ester moiety at C-10, as determined by correlations in the HMBC and NOESY spectra. Compound **4**, unlike other daucanes isolated from *L. latifolium*, features an additional α-oriented hydroxy group at position C-9, as evidenced by the crosspeaks of the β-oriented H-9 (δH 2.94, *d* 5.5 Hz) and the protons of Me-14 (δH 1.44, *s*) and Me-15 (δH 1.39, *s*) in the NOESY spectrum. Finally, compound **4** was assigned a structure of the novel laserpitinol ester: 10α-acetoxy-2β,6α-angeloyloxy-dauca-4β,8α,9α-triol (**Fig. 1**). Spectral data of compound **6** revealed this compound is a novel isomer of a monoangeloyloxy-laserol (**Fig. 1**), isolated previously from the *L. latifolium* underground parts (Holub et al., 1967a), as the HMBC spectrum of this novel isomerindicated the presence of an angeloyloxy group at position 6α instead of 10α. Compound **7** was featuring characteristic chemical shifts (1H and 13C NMR) of a laserpitin analogue (**Fig. S7-S9**, Supplementary material). However, this compound features just one angeloyloxy group at position 6α and the hydroxyl group at 10α is esterified by a threo isomer of 2,2-dihydroxyl-isopentanoic acid, an acid of which the esters are typically present in alkaloids (Jenett-Siems et al., 1993). Thus, **7** was characterised as10α-deangeloyloxy-10α-(2,3-dihydroxyl-2-methyl-butanoiloxy)-laserpitin (**Fig. 1**).

*Daucanes inhibit NF-κB- and AP-1-dependent gene expression*

Despite of the ethnopharmacological evidence that the *L. latifolium* underground parts were used to treat disorders connected to chronic inflammation, daucane esters as its major constituents were not yet characterized for their anti-inflammatory activity. Therefore, we screened isolated daucanes from the roots and rhizomes of *L. latifolium* for their ability to inhibit NF-κB- and AP-1-driven pro-inflammatory pathways in the A549 human lung epithelial cell line. We used this cell model as a research tool to discover novel anti-inflammatory agents, especially the ones that may be able to act anti-inflammatory without activating the conserved mechanisms typically responsible for metabolic side effects associated with glucocorticoid therapy. The inhibition of a TNF-induced stably integrated recombinant NF-κB-dependent reporter, as well as the inhibition of PMA-induced stably integrated recombinant AP-1-dependent reporter gene was followed in presence of daucane esters (**1-8**) at three concentrations (60, 30 and 10 μM), which enabled monitoring of the concentration-dependent specific gene inhibition. The effects of laserpitin were reported elsewhere (Popović et al., submitted). Control groups represented cells that received an equivalent volume of ethanol, as the solvent in which compounds/DEX were dissolved. Dexamethasone, as a synthetic GR agonist (1 μM) was used as a reference drug, since it is known to strongly inhibit NF-κB- and AP-1-dependent pathways. Cytotoxicity was reported previously for daucanes of similar structures (Alkhatib et al., 2008, Dall’ Acqua et al., 2011 and 2014, Jabrane et al., 2010, Poli et al., 2005). To monitor potential cytotoxic effects of compounds **1-8**,constitutive luciferase production was assayed in the hereto specially designed Neo-Luc reporter cell line to evaluate whether or not our compounds affect cell viability. The results of the inhibition pro-inflammatory transcription factor activities are given in Table 1 for the NF-κB assay and in Table 2 for the AP-1 assay. The results are given as the percentage of reporter gene activity after treatment with the highest concentration of the compound (60 μM) compared to the maximal effect of pro-inflammatory agent in the control group which is set at 100%. Notably, the daucanes featuring the higher number of ester moieties such as desoxodehydrolaserpitin (**3**), acetyldesoxodehydrolaserpitin (**8**) and a novel laserpitinol analogue featuring an extra α-oriented hydroxyl group at C-9 (**4**) exerted the highest activities. In presence of compounds **3**, **8** and **4**, NF-κB-dependent reporter gene activity in A549 cells was decreased to 52.69 ± 9.86%, 53.25 ± 9.34% and 34.04 ± 6.75%, respectively, as compared to a solvent group that received just TNF (200 IU/ml, set at 100%). The concentration-dependent repression of NF-κB-driven reporter gene activity by compounds **3**, **4** and **8** is shown in Fig. 2A. For the most active compound **4**, the IC50 in this assay was 47.04 μM. A similar outcome was observed for the PMA-induced AP-1 reporter gene stably integrated in the A549 cell line: the highest effect was observed by adding 60μM of desoxodehydrolaserpitin (**3**), acetyldesoxodehydrolaserpitin (**8**) and a novel daucane ester **4**, decreasing the reporter gene activity to 58.16 ± 0.43%, 46.10 ± 5.06% and 34.04 ± 4.36%, respectively, as compared to the solvent group induced by PMA (20nM) being set at a 100% AP-1 reporter gene activity. The concentration-dependent effects of **3**, **4** and **8** are shown in Fig. 2B.10α-Acetoxy-2β,6α-angeloyloxy-dauca-4β,8α,9α-triol (**4**) was again the most active compound in the reporter gene assay targeting the AP-1-driven pro-inflammatory pathway, and its IC50 value was 36.37 μM which represents a remarkable activity and a good starting point for further investigation of the mode of action. Notably, two daucane esters **1** and **2** that were featuring one hydroxylation/ester moiety less than the desoxodehydrolaserpitin analogues (**3**, **8**) and the highly esterified laserpitinol derivative (**4**), showed milder effects in the reporter gene assays (reporter gene activities of 63.15 ± 7.32% and 61.26 ± 1.68% for the NF-κB reporter gene and 65.54 ± 3.59% and 66.89 ± 3.72% for the AP-1 reporter gene, with compounds **1** and **2**, respectively). Laserpitin analogues **5** and **7** and monoangeloyloxy-laserol **6** showed the weakest effects both in NF-κB and AP-1 reporter gene assays (Tables 1 and 2). To exclude the cytotoxic effects, a parallel measuring of the luciferase activity in the NeoLuc cell line as well as a monitoring of visual toxicity-induced changes in morphology of the cells observed under the microscope was performed. For the most prominent daucanes, the toxicity was negligible (results presented as Fig. S11 in Supplementary material) as the cell viability remained higher than 90% even at the highest concentration tested (60 μM).

As reported previously, there is insufficient data on the anti-inflammatory potential of sesquiterpenes from the daucane class. Up to now in only one study effects of three newly isolated daucanes longiferone A, B and C isolated from the rhizomes of *Boesenbergia longiflora* were evaluated for their *in vitro* anti-inflammatory activity. Two of those novel daucanes longiferone B and C were able to inhibit NO but not TNF-α production in a LPS-stimulated murine macrophages RAW264.7 cell line (Sudsai et al., 2014).

*Daucanes inhibit GRE-driven gene expression*

As mentioned before, detrimental side effects of glucocorticoid treatment, especially with respect to diabetes, which limit the therapy duration and urge the search for new alternatives, are related to the activation of glucocorticoid response element (GRE)-dependent genes (Sundahl et al., 2015). Compounds able to interfere with this GRE-dependent pathway may be interesting to combine with classic glucocorticoids, to lower the dose of the latter drug and at the same time to alleviate gluconeogenic side effects. Therefore, when investigating the therapeutic potential of daucane esters from the underground parts of *L. latifolium* apart of repression of pro-inflammatory NF-κB and AP-1 pathways, we wanted to test the effect of compounds **1-8** on a DEX-induced GR-driven GRE-dependent reporter gene activity in A549 cells, which endogenously express GR. This assay aims at testing whether or not daucane compounds may exhibit a potential competitive effect on GR-mediated GRE-driven gene expression. This feature could open new therapeutic possibilities as a co-application with compounds repressing DEX-induced GRE elements especially in chronic diseases, which is expected to improve the therapeutic index i.e. combining a favorable therapeutic outcome with a reduction of metabolic side effects (De Bosscher et al., 2015) or to reduce expression of pro-tumorigenic genes (Chen et al., 2015). DEX was tested at two concentrations, 0.1 and 0.01 μM, using the GRE-driven reporter gene stably integrated in A549 lung epithelial cells. The compounds were tested at 60 and 30 μM. Obtained results are presented in Table 3. Remarkably, all tested entities, except for **6**, had the capacity to at least mildly reduce DEX-induced GRE-driven reporter gene activities. Compounds **2**, **3**, **4** and **8** showed the most outspoken competitive effect. The most potent compound was again novel daucane 10α-acetoxy-2β,6α-diangeloyloxy-dauca-4β,8α,9α-triol (**4**). At 60 μM, it reduced GRE-driven promoter activities to 44.88% ± 13.81% and 34.80 ± 5.79% when GR was triggered by 0.1 and 0.01 μM DEX (the activity of DEX at these concentrations in the presence of solvent alone was considered as a 100% of activity). Also, daucanes featuring ester moieties at position C-2, C-6 and/or C-10, such as paliin (**2**), desoxodehydrolaserpitin (**3**) and acetyldesoxodehydrolaserpitin (**8**) exerted a statistically significant reduction of DEX-induced GRE-driven reporter gene activities in A549 cells. Therefore, some daucane esters featuring Δ8,9 unsaturation, and esters at positions 2β, 6α and/or 10α, and specially2β-esterified laserpitinol analogues may represent further candidates that may tweak GR responses into more selective pathways and may be further brought to therapeutics that might, solely or in combination therapy with dexamethasone, act anti-inflammatory while dampening known metabolic side effects (De Bosscher et al., 2016).

*Daucanes inhibit concomitant CCL-2 production*

In order to strengthen our findings, endogenous CCL-2 pro-inflammatory chemokine levels in cells treated with the most promising daucanes were tested in inflamed vs. non-inflamed control groups. The CCL-2 chemokine was chosen as it represents an inflammatory mediator found in many T-cell mediated inflammatory conditions as it acts as chemoattractant for lymphocytes and monocytes (Woldemar Carr et al., 1994). Compounds **3**, **4** and **8** (30 μM) as well as DEX (1 μM) were administered to basal A549 cells and concomitant protein production was determined via ELISA. All three compounds (**Fig. 3**) significantly inhibited CCL-2 chemokine production upon treatment with TNF (200 IU/ml) to concentrations of 4.05 ± 0.57 ng/ml, 4.11 ± 0.11 ng/ml and 3.60 ± 0.12 ng/ml after adding 30 μM of compounds **3**, **4** and **8**,respectively vs. 6.48 ± 0.5 ng/ml of CCL-2 in the TNF-only control group. Dexamethasone, used as a control drug, inhibited CCL-2 production to 1.99 ± 0.51 ng/ml.

*Daucanes inhibit CCL-2, IL-1β and IL-6 mRNA expression*

Since the most active daucanes were able to suppress protein production of an anti-inflammatory mediator, CCL-2, to a similar extent as what was observed for the reporter gene assays, we wondered whether this would also be the case for a transcriptional outcome of other mediators of inflammation. Hereto, we tested the effect of the three most active compounds **3**, **4** and **8** on mRNA expression levels upon inflammation (TNF) of CCL-2, IL-1β and IL-6 in A549. The results are shown in **Fig. 4**. Compounds **3** and **8** mildly inhibited (30 μM) mRNA expression of the TNF-induced pro-inflammatory genes CCL-2, IL-1β and IL-6 (data not shown), however the effect of 10α-acetoxy-2β,6α-angeloyloxy-dauca-4β,8α,9α-triol (**4**) was the most pronounced and comparable to the effect of DEX (1 μM) (**Fig. 4**). The novel laserpitinol ester (**4)** and dexamethasone (DEX) showed a statistically significant repression of genes coding for CCL-2 and IL-6 (**Fig. 4A** and **4C**), while the attenuation of IL-1β mRNA levels was not significant (Fig. **4B**), indicating a target gene-dependent effect of the compound (inflammatory mRNA). In a study on constituents of *Boesenbergia longiflora* rhizomes, it was shown that daucane esters may indeed have atarget gene-dependent effect on expression levels. Longifeone B showed a concentration-dependent inhibition of iNOS and COX-2 mRNA levels while it did not influence TNF-α mRNA (Sudsai et al., 2014). In this study, CCL-2, IL-1β and IL-6 are chosen as typical markers of inflammation in humans (Westberg et al., 2016; Woldemar Carr et al., 1994). Also, specific inducible genes levels such as the ones coding for IL-6 is mediated by NF-κB and AP-1 pathways, and DEX is known to efficiently block both pathways (De Bosscher et al., 2014).

*Daucane mildly stimulates the GRE-driven anti-inflammatory gene GILZ, but not DUSP1*

The protein products of some of the GRE-mediated genes, such as for instance Glucose-6-phosphatase in liver, contribute to glucocorticoid-induced side effects, whereas other GRE-driven genes coding for proteins such as DUSP1 and GILZ rather have a role in resolving inflammation (Clark and Belvisi, 2012; Ronchetti et al., 2015). Because of the observed competitive effect of daucanes on GRE reporter activity, which could indicate interaction with GR activity, we wondered about the capacity of daucanes to independently stimulate GRE-mediated gene expression. Hereto, we tested the effect of the most active compound **4** (30 μM) on mRNA expression levels of DUSP1 and GILZ in absence and presence of an inflammatory stimulus (TNF) in A549 cells. DEX (1 μM), a known inducer of GILZ and DUSP1 expression was used as reference compound. The results are shown in **Fig. 5A** and **B**. As expected, both GILZ and DUSP1 expression levels were strongly upregulated by DEX as compared to the control (solvent) group. Surprisingly, up-regulation of GILZ by compound **4** was observed, yet only in non-inflamed (-TNF) cells (**Fig. 5A**). This may point to interference by TNF signaling on the GR-modulatory capacity of compound **4**, as this compound alone does allow for a small proportion of GRE-driven expression of this particular anti-inflammatory gene. The ability of TNF to lower DEX-induced mRNA levels of both GILZ and DUSP is a known phenomenon and believed to contribute to GC resistance (Van Bogaert et al., 2010). On the other hand, effects of compound **4** on DUSP1 levels were absent in both inflamed and non-inflamed cells (**Fig. 5B**). This difference may either point to a target-specific gene regulatory mechanism that may be ascribed to slightly different GRE sequences in both gene promoters, or alternatively, even to a GR-independent effect of compound **4** on GILZ mRNA expression. Further experiments are needed to resolve the underlying mechanism. Given the fact that the GRE-driven reporter gene assay (Table 3) showed that daucanes can interfere with classic GRE-driven gene expression, we postulate that a combination between DEX and daucanes will probably lead to a biological profile that is still anti-inflammatory with respect to cytokine inhibition, but may be less performant at the level of anti-inflammatory gene expression. However, this profile would still be preferable in a chronic treatment context, where one would want to avoid GRE-driven gene expression of gluconeogenic enzymes.

**Conclusions**

In the current study, rare and novel daucane esters from the underground parts (roots and rhizomes) of laserwort, *L. latifolium*, featuring esters at positions 2β, 6α and/or 10α, and especially a 2β-esterified laserpitinol analogue were shown to efficiently inhibit NF-κB and AP-1 transcriptional pathways in a human lung epithelial cell line. The most active entities significantly inhibited concomitant protein excretion and pro-inflammatory gene expression. Moreover, these compounds may modulate GR-dependent pathways to achieve a so-called dissociative profile i.e. whilst attenuating inflammation, they also inhibit Dexamethasone-induced GRE-response element activation, which is associated to metabolic side effects of glucocorticoids. As for GRE-driven anti-inflammatory gene expression, from the current data it is clear that compound **4** mildly supports GRE-driven mRNA expression of GILZ, not DUSP1, in A549 cells. These results may point to gene-specific differences for different GREs, in line with previous findings for DEX by Meijsing and colleagues (2009), or may be explained by alternative mechanisms. It can be concluded that daucane esters represent an efficient new class of anti-inflammatory agents, which could be developed in a new anti-inflammatory therapeutic regimen and used either as a therapy replacement or co-therapy with glucocorticoids.

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**Conflict of interest**

The authors declare that there is no conflict of interest.

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**Table legends**

**Table 1**

Daucanes of *L. latifolium* can inhibit TNF-induced NF-κB-dependent gene transcription in A549 cells. Inhibitory effect of compounds 1-9 in applied in highest concentration (60 μM) as well as Dexamethasone DEX (1 μM) are expressed as a percent of maximal reporter gene levels induced by TNF in presence of solvent alone (set at 100% of activity) are given as mean of three independent biological replicates ± SEM.

**Table 2**

Daucanes of *L. latifolium* can inhibit PMA-induced AP-1-dependent gene transcription in the A549 cells. Inhibiting activity of compounds 1-9 (60 μM) as well as Dexamethasone DEX (1 μM) are expressed as a percent of maximal effect (reporter gene levels) achieved by solvent control group alone induced by PMA (set at 100%). Results are shown as mean of three independent biological replicates ± SEM.

**Table 3**

Effects of compounds **1-8** (60 and 30 µM) on DEX-induced GRE-driven reporter gene activities in the A549 lung epithelial cell line. Reporter gene levels induced by DEX at two concentrations (0.1 and 0.01 µM) in presence of solvent alone were each set at 100% of Activity. Compound **6** showed little to no effect this assay (<10% of inhibition).

**Figure legends**

**Fig. 1.** Structures of daucane esters isolated from the roots and rhizomes of medicinal species *L. latifolium*.

**Fig. 2.** Multiple ester containing daucanes inhibit NF-κB- and AP-1-driven gene expression in a concentration dependent manner. Effect of desoxodehydrolaserpitin (**3**), 10α-acetoxy-2β,6α-angeloyloxy-dauca-4β,8α,9α-triol (**4**) and acetyldesoxodehydrolaserpitine (**8**) in A. NF-κB and B. AP-1 gene reporter assays applied at three concentrations (10, 30 and 60 μM). Results are expressed as percentage of activities as compared to the maximal activity achieved by TNF (200 IU/ml) in a control (solvent group) that is set at 100%. Results are shown as a mean value ± SEM of three independent replicates. Statistical significances between the control group (solvent - NI) treated with TNF (200 IU/ml) and groups treated with Daucanes **3**, **4** and **8** or dexamethasone DEX were calculated for the raw data (before expressing them as % of maximal activity of TNF) by One-way ANOVA using Dunett’s correction (significance levels \*p≤0.05; \*\*p≤0.01; \*\*\*p≤0.001).

**Fig. 3.** Effect of desoxodehydrolaserpitin (**3**), 10α-acetoxy-2β,6α-angeloyloxy-dauca-4β,8α,9α-triol (**4**) and acetyldesoxodehydrolaserpitine (**8**) at a concentration of 30 μM on CCL2 (concentration in ng/ml) production in basal A549 cells. Results are shown as a mean value ± SEM of at least three independent replicates. Statistical significances between the control group (solvent - NI) treated with TNF (200 IU/ml) and groups treated with daucanes or dexamethasone DEX were calculated by One-way ANOVA using Dunett’s correction (significance levels \*p≤0.05; \*\*p≤0.01; \*\*\*p≤0.001).

**Fig. 4.** Compound **4** (30 μM) inhibits pro-inflammatory gene expression (**Fig. 4: A** CCL-2, **B** IL-1β and **C** IL-6 gene) in a target gene dependant manner in A549 cells. Results are shown as a mean value ± SEM of three independent replicates. Statistical significances between the control group (solvent - NI) treated with TNF (200 IU/ml) and groups treated with Compound **4** or dexamethasone DEX (1 μM) were calculated by One-way ANOVA using Dunett’s correction (significance levels \*p≤0.05; \*\*p≤0.01; \*\*\*p≤0.001). Rel.: Relative.

**Fig. 5.** Compound **4** (30 μM) mildly up-regulates GRE-mediated anti-inflammatory gene expression (**Fig. 5**: **A** GILZ and **B** DUSP1 gene) in a target gene-dependent manner in A549 cells. Results are shown as a mean value ± SEM of three independent replicates. Statistical significances (calculated on the raw datasets) between the control group (solvent - NI) treated with TNF (200 IU/ml) and groups treated with Compound **4** or dexamethasone DEX (1 μM) were calculated by One-way ANOVA using Dunett’s correction (significance levels \*p≤0.05; \*\*p≤0.01; \*\*\*p≤0.001). Rel.: Relative.



**Graphical abstract**

The most active compound **4** (30 μM), anovel laserpitinol ester 10α-acetoxy-2β,6α-angeloyloxy-dauca-4β,8α,9α-triol inhibits pro-inflammatory gene expression in a target gene dependant manner in A549 cells. Statistical significances between the control group (solvent - NI) treated with TNF (200 IU/mL) and groups treated with Compound **4** or dexamethasone DEX (1 μM) were calculated by One-way ANOVA using Dunett’s correction (significance levels \*p≤0.05; \*\*p≤0.01; \*\*\*p≤0.001). Rel.: Relative.

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