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Cytotoxic Activity of *Laserpitium latifolium* L. Extract and Its Daucane and Phenylpropanoid Constituents

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Abstract: In the search for novel cytotoxic agents, sesquiterpenoids and phenylpropanoids have provided interesting lead compounds. From the chloroform extract of the underground parts of *Laserpitium latifolium*, the daucane sesquiterpenoids laserpitin and acetyldesoxodehydrolaserpitin, and the phenylpropanoids laserin and latifolon were isolated as the major compounds. Acetyldesoxodehydrolaserpitin is identified for the first time in the genus *Laserpitium*. Using a MTT and a sulforhodamine B (SRB) assay, the cytotoxic and antiproliferative activity of the extract and compounds laserpitin, acetyldesoxodehydrolaserpitin and laserin was tested in two closely related human breast adenocarcinoma cell lines, ie. the invasive MCF 7/6 and the non-invasive MCF 7/AZ. The IC_{50} values of the extract were in the range of 184.72 – 397.16 μg/mL, with the most potent effect observed in the MTT test on the MCF 7/6 line. Among the tested compounds, acetyldesoxodehydrolaserpitin exerted a most potent, concentration-dependent effect (IC_{50} values of 0.60 and 0.51 μM in the MCF 7/6 cell line, and 2.29 μM and 31.87 μM in the MCF 7/AZ cell line in the MTT and SRB test, respectively). The effect of laserin was more pronounced in the MTT test (IC_{50} of 4.57 and 2.46 μM in the MCF 7/6 and MCF 7/AZ, respectively).

Keywords: Daucane esters; phenylpropanoids; *Laserpitium latifolium;* cytotoxic activity.

1. Plant Source

Laserpitium latifolium L. (Apiaceae) is widely distributed European species, whose underground parts (roots and rhizomes), known as Radix Gentianae albae, were used in traditional medicine for their sharp and bitter taste for preparing tonics for refreshing and strengthening, as well as for their diuretic and carminative properties [1,2]. The underground parts of *L. latifolium*, used in this investigation were collected at Mt. Gučevo (West-Serbia) in October 2008. The plant material was collected and identified by Dr. Marjan Niketić, and a voucher specimen was deposited at the Natural History Museum in Belgrade under the accession number ko03102008.

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2. Previous Studies

Previous chemical investigations of the extracts of underground parts of *L. latifolium* revealed the presence of daucane derivatives such as laserpitin [3,4], phenylpropanoid derivatives such as latifolon [4], while from extracts of herb were isolated sesquiterpene lactones of the guaianolide type [5].

Daucane derivatives are a relatively small group of sesquiterpenoids commonly isolated from Apiaceae species [6]. Some daucane esters exert cytotoxic activity on several human cancer cell lines [7]. The phenylpropanoid laserin was isolated and identified as the bitter compound present in carrot, *Daucus carrota* L., and has been shown to exhibit cytotoxic activity against HL-60 cells [8].

3. Present Study

The underground parts of *L. latifolium* (631.96 g) were extracted twice with $CHCl_3$ for 48 h at room temperature, at a ratio of 100 g of powdered material in 1 L of $CHCl_3$. The collected filtrates were concentrated under vacuum to obtain a dark brown, gummy extract (31.78 g). Part of the crude $CHCl_3$ extract (4.51 g) was solubilized in MeOH and filtrated in order to remove waxes and lipophilic compounds. The obtained MeOH soluble fraction was dried under reduced pressure (4.46 g) and further eluted with a stepwise gradient system with increasing polarity of a solvent mixture composed of hexane-EtOAc (from 5:1 to pure EtOAc), followed by EtOAc-MeOH (from 1:1 to pure MeOH). In total 120 fractions were collected, in a volume of 20 mL each. Separation was followed by TLC analyses using vanillin-sulphuric acid for derivatization. The total extract and selected fractions were further analyzed by HPLC using a gradient system optimized for terpenoids. The mobile phases consisted of 0.025% HCOOH in H_2O (solvent A) and 0.025% HCOOH in 50 : 50 v/v of H_3CN and MeOH (solvent B). The elution program was 0-3 min isocratic 35.0% A (65% of B), 3-25 min linear gradient from 35 to 15% of A (85% of B), 25–28 min linear gradient from 15 to 0% of A (100% of B), 28–30 min isocratic 0% A (100% of B), 30-32 min linear gradient 0 to 35% A (65% of B) and 32-35 min isocratic 35% A (65 of B). The flow rate was 0.7 mL/min; the temperature was set at 35 °C; an injection volume of 20 μL was applied and a detection wavelength of 225 nm was used.

From the $CHCl_3$ extract of *L. latifolium* underground parts, four compounds were isolated as the main constituents: daucane esters laserpitin and acetyldesoxodehydrolaserpitin and phenylpropanoids laserine and latifolone. The sturucture of all isolated compounds was elucidated by 1D (^{1}H NMR, ^{13}C NMR) and 2D (gHSQC, gHMBC and COSY) NMR analyses, and further confirmed by high resolution MS.

Latifolon (λ_{max}=219; 298 nm; R_t=6.47 min) was isolated as an amorphous solid, with the molecular formula $C_{11}H_{12}O_4$ as assigned by HR-MS (*m/z* molecular ion peak at 209.0826 (calcd for $[M + H]^+$, 209.0736). Also its ^{1}H and ^{13}C NMR spectra were corresponding to the structure of latifolon, previously isolated from *L. latifolium* root [4].

Laserpitin (λ_{max}=223 nm; R_t=18.22 min) was isolated as a white crystalline powder and with molecular formula $C_{25}H_{38}O_7$, assigned on the basis of the *m/z* molecular ion peak at 451.2759 (calcd for $[M + H]^+$, 451.2690) in HR-MS. 1D and 2D NMR experiments allowed to identify characteristic patterns of two angelic acid esters bounded to alcohol laserol (Figure 1). Interestingly, laserpitin was the first isolated daucane derivative [6] whose structure, due to the complexity, was finally established after several revisions [3].

Acetyldesoxodehydrolaserpitin (λ_{max}=223 nm; R_t=25.84 min) was isolated as a sticky, colourless liquid. The molecular formula $C_{27}H_{40}O_7$ was assigned by HR-MS with *m/z* molecular ion peak at 477.2843 (calcd for $[M + H]^+$, 477.2847) and *m/z* for the pseudomolecular ion peak at 499.2658 (calcd for $[M + Na]^+$, 499.2666). The NMR spectra allowed characteristic patterns for two angeloyl and one acetyl ester moieties, bound to the daucane skeleton. The ^{1}H NMR spectrum matches with that of the daucane ester acetyldesoxodehydrolaserpitin that was previously isolated only from the roots of *Ferula tingitana* L. [9].

Laserin (λ_{max}=223 nm; R_t=20.86 min) was retrieved as a sticky, colourless liquid, with the molecular formula $C_{21}H_{26}O_7$ derived from the pseudomolecular ion peaks at *m/z* 413.1584 and

429.1335 (calcd for $[M + Na]^+$, 413.1600 and for $[M + K]^+$, 429.1300). ^1H and ^{13}C NMR data were matching the previously reported spectra for laserin [8], isolated from *L. archangelica* Wulf. [10] and *L. garganicum* (Ten.) Bertol. [11].

In the system for terpenoids, using isolated compounds as external standards, contents of compounds were quantified, and it was determined that concentrations of laserpitin, acetyldesoxodehydrolaserpitin, laserin and latifolon were respectively 17.69, 1.02, 0.72 and 0.64 mg per g of the dry weight of *L. latifolium* underground parts.

The cytotoxic and antiproliferative activities of $CHCl_3$ *L. latifolium* extract and isolated daucanes laserpitin and acetyldesoxodehydrolaserpitin and the phenylpropanoid laserin were investigated using two *in vitro* colorimetric assays namely: MTT [12] (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), for detecting the number of living, metabolically active cells, and SRB [13] (sulforhodamine B) for measuring the total cellular protein content. Activity was tested on the two human breast adenocarcinoma cell lines: MCF 7/6 and MCF 7/AZ, invasive and non-invasive type, respectively. The *L. latifolium* $CHCl_3$ extract and the pure compounds were tested at five concentrations between the following ranges: 7.5-750 μg/mL for the extract and 0.5-100 μM for compounds. Vinblastine sulphate was used as a reference compound at five concentrations ranging from 0.1 to 40 nM. The results expressed as IC_{50} values (extract concentration required to inhibit 50% of the cell growth) are given in Table 1. The negative controls were carried out by replacing the test compounds by corresponding volumes of medium and these results were considered to represent 100% of cell growth.

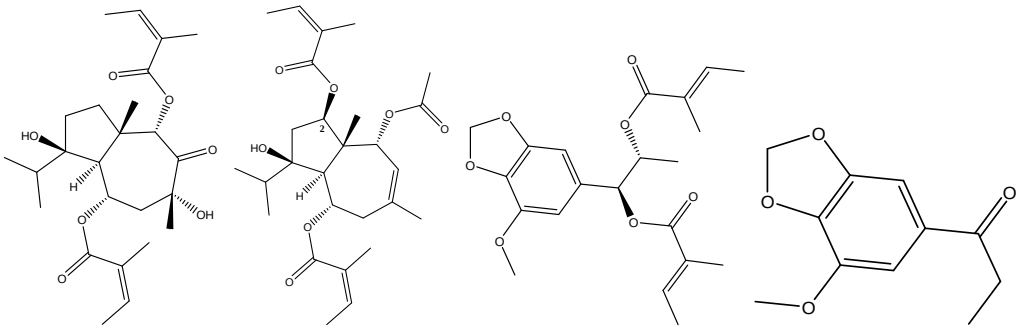
The extract showed a concentration dependent cytotoxic and/or antiproliferative activity with IC_{50} values ranging from 184.72 to 397.16 μg/mL, and the highest effect was achieved in the MTT test on the invasive MCF 7/6 cell line (Table 1).

Among the compounds tested, acetyldesoxodehydrolaserpitin (Figure 1) was the most active compound, especially on highly invasive MCF 7/6 cell line in both assays tested. Determined IC_{50} values were in the low micromolar range (0.51 and 0.60 μM at MCF 7/6 line in the SRB and the MTT test, respectively and 2.29 μM on MCF 7/AZ in the MTT test), except IC_{50} value of 31.87 μM in SRB test at MCF 7/AZ cell line, where exerted cytotoxic and/or antiproliferative activity was weaker. The cell growth inhibition caused by acetyldesoxodehydrolaserpitin (5 μM) and the reference compound vinblastine sulphate (10 nM) are given in the Figure 2. The results are expressed as the mean values ± SD of four independent replicates. Some compounds of the class of daucanes were previously shown to, at least in part, contribute to the cytotoxic activity of several *Ferula* species, such as elaeochytrin A, 6-antraniloyl daucane ester isolated from *F. elaeochytris* Korovin that was the most active compound in a MTT assay against two resistant cell lines of human (K562R) and mouse ($DA1\text{-}3b/M2^{BCR\text{-}ABL}$) leukemia ($IC_{50}$ values 12.4 and 7.8 μM, respectively) [14]. In a panel investigation of the cytotoxicity of 16 daucane esters isolated from two *Ferula* and one *Ferulago* species on seven human tumor cell lines, 14 of them showed activity against at least one of the tumor cell lines [7]. Pallinin, a 6α,10α diangeloyl daucane ester, was the most active compound with IC_{50} values in the lower molecular range against six cell lines tested [7]. Structure-activity relationship investigation showed that the β-orientation of ester group at position C(2) strongly enhances the cytotoxic activity [7]. Furthermore, the presence of an α,β-unsaturated ketone ("enone") groups in side chains of sesquiterpenes and sesquiterpene lactones increases the cytotoxicity toward tumor cells [15]. Acetyldesoxodehydrolaserpitin is a tri-ester, with two angeloyloxi groups featuring an "enone" system one of which has *β*-orientation at position C(2), thereby combining several structural factors contributing to the observed potent cytotoxicity in human breast adenocarcinoma cell lines.

Laserin exerted a concentration dependent effect in both tests on both cell lines, with the most potent activity in the MTT test for both cell lines (IC_{50} values 4.57 and 2.46 μM on MCF 7/6 and MCF 7/AZ, respectively). This compound and specially its erythro isomer 2-epilaserin that was isolated from carrot were characterized as cytotoxic phenylpropanoids in the human acute promyelocytic leukemia cell line HL-60 [15].

Our results suggest that both the daucane esters and phenylpropanoid laserin may be, at least in part, responsible for the demonstrated cytotoxic and antiproliferative activity of the investigated *L. latifolium* extract. Among the isolated compounds, acetyldesoxodehydrolaserpitin, whose biological

activity had not been tested before, was identified as the most potent cytotoxic and/or antiproliferative agent, especially in the invasive MCF 7/6 cell line.



1 **2** **3** **4**

Figure 1. Structure of daucane esters laserpitin (**1**) acetyldesoxodehydrolaserpitin (**2**) and phenylpropanoids laserin (**3**) and latifolon (**4**) isolated from the $CHCl_3$ extract of *L. latifolium* underground parts.

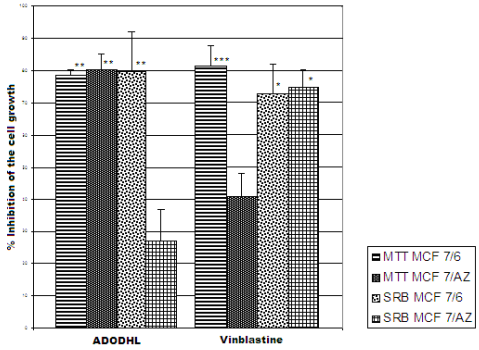


Figure 2. Percent of inhibition of the cell growth of the two human breast carcinoma cell lines MCF 7/6 and MCF 7/AZ in MTT and SRB tests, in comparison to the negative controls after application of 5 μM of acetyldesoxodehydrolaserpitin (ADODHL) and 10 nM of vinblastine sulphate as a reference compound. Results are expressed as the mean values ± SD of four independent replicates. Statistical significance of inhibition was determined in comparison to control groups at significance level * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Table 1. *In vitro* cytotoxic activity expressed as IC_{50} values on MCF 7/6 and MCF 7/AZ cell lines for *L. latifolium* $CHCl_3$ extract, compounds and reference compound (vinblastine sulphate) in MTT and SRB assays.

Compound	MTT		SRB	
	MCF 7/6	MCF 7/AZ	MCF 7/6	MCF 7/AZ
1[a]	31.80	87.13	>100	>100
2[a]	0.60	2.29	0.51	31.87
3[a]	4.57	2.46	62.04	7.36
Vinblastine[a]	5.98×10^{-3}	15.41×10^{-3}	4.11×10^{-3}	3.66×10^{-3}
Extract[b]	184.72	208.94	208.85	397.16

[a] *IC_{50} values for laserpitin (**1**), acetyldesoxodehydrolaserpitin (**2**), laserin (**3**) and , vinblastine (reference compound) are given in μM;*
[b] *IC_{50} values for L. latifolium $CHCl_3$ extract are given in μg/ml.*

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