Anticancer Effect of the Iridoid Glycoside Fraction from *Dipsacus fullonum* L. Leaves

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Abstract

Species of *Dipsacus* have been widely used in folk medicine for their neuroprotective, antiosteoporotic, antioxidative, anticomplementary, and antibacterial activities. However, there has been but a limited amount of research on the anticancer effect of one of the most popular representatives of this genus, *D. fullonum*. Also, the cytotoxic activity has not yet been investigated of the constituents of *D. fullonum* leaves. The purpose of this study was to evaluate the cytotoxic activity of the bis-iridoid glycosides isolated from *D. fullonum* leaves against murine fibroblast NIH/3T3, mouse melanoma B16F10, HeLa human cervical cancer, human breast cancer MCF7 and MDB-231 cells. The bis-iridoids, obtained by chromatographic fractionation of the extract of *D. fullonum* leaves, were characterized by thin-layer chromatography and high-performance liquid chromatography-mass spectrometry (HPLC-MS)/MS analysis. The cytotoxicity of the iridoid fraction was evaluated by WST-1 assay, and the number of dead cells was determined by the propidium iodide test. HPLC-MS/MS analysis showed the isolated bis-iridoid fraction to consist mainly of sylvestroside III and/or sylvestroside IV. This fraction was applied to cell cultures and kept for 48 and 72 hours. The results demonstrated that the iridoid glycosides had a differential ability to induce cell death in normal and cancer cells. The study confirmed that the bis-iridoids extracted from *D. fullonum* leaves had a selective cytotoxic effect on human breast cancer cell lines MCF7 and MDB-MD-231, while their cytotoxic effect on noncancer cells was low.

Keywords

*Dipsacus fullonum*, iridoid glycosides, bioactivity, anticancer activity, HPLC-MS

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Through the ages herbal extracts have widely been used in folk medicine to treat different diseases, while modern phytochemical and pharmacological investigations have provided further knowledge about the use of medicinal plants. The genus *Dipsacus* comprises 15 species that are distributed throughout Europe, West Asia, and North Africa,¹ and are popular herbal remedies used in folk medicine. Of the *Dipsacus* species, *D. asper* and *D. asperoides* roots and seeds have been investigated most thoroughly. They have long been used to treat low back pain, traumatic hematoma, bone fractures, and inflammation.²,³ The extracts, as well as isolates, of selected *Dipsacus* species have been shown to exhibit a variety of bioactivities, including neuroprotective,⁴ antiosteoporotic,⁵ antioxidative,² anticomplementary,⁶ and antibacterial.⁷ In addition, these species have been reported to have a therapeutic effect on allergic asthma⁸ and different skin diseases.¹¹-¹⁵ A recent study revealed dozens of bioactive compounds in *Dipsacus* species, including triterpenoids, triterpenoid saponins, iridoid glycosides, phenolic acids, and alkaloids.¹⁶

Despite the fact that *Dipsacus* has a long folk history for the treatment of cancer,¹³ data on its cytotoxic and antitumor activities are scarce. There have been only a few papers on the antiproliferative activities of its whole root extract,¹⁷ and the isolated active compounds, such as water-soluble polysaccharide (MW 16 kDa),¹⁸ indole alkaloids,¹⁹ and saponins.²⁰-²²

Iridoids, another type of bioactive compounds of plant origin, are a large group of cyclopentano [c] pyran monoterprenoids biosynthesized from isoprene. These iridoids and their glycosides are constituents of a large number of plant families, including *Dipsacus* species. The above-mentioned compounds have a biogenetic and chemotaxonomic importance since they provide a structural link between terpenes and alkaloids. A
significant antitumor activity of iridoid glycosides has been reported by a number of researchers.\textsuperscript{23-28} To date, about 30 iridoids have been isolated from \textit{D. asper}, \textit{D. laciniatus}, \textit{D. fullonum}, \textit{D. japonicus}, and \textit{D. ferox},\textsuperscript{16,29} but their cytotoxic effect has been evaluated by only a few researchers.\textsuperscript{30,31} The wide distribution and remarkable pharmacological activities of \textit{Dipsacus} species indicate their potential for the discovery and development of new natural drugs. Previously, the leaves and root extract of \textit{D. fullonum} were investigated only for the inhibition of \textit{a}-amylase,\textsuperscript{32} while the descriptions of its therapeutic properties can mostly be found in encyclopedias.\textsuperscript{12,15} The applicability of \textit{D. fullonum} to the treatment of cancer in folk medicine has been mentioned in some publications.\textsuperscript{13,14} This study investigated the secondary metabolites of \textit{D. fullonum} leaves with the aim of searching for compounds with anticancer activity. Therefore, the iridoid glycoside fraction of the methanolic extract was characterized, and its in vitro cytotoxicity established against murine fibroblast NIH/3T3, mouse melanoma B16F10, HeLa human cervical cancer, and human breast cancer MCF7 and MDB-MB-231 cells.

\textbf{Results and Discussion}

\textit{Separation and Identification of the Bis-Iridoid Glycoside Fraction}

For qualitative evaluation, all the collected fractions were analyzed by thin-layer chromatography (TLC), using UV irradiation at 254 and 312 nm to visualize the presence of compounds of interest. The irradiation wavelength of 312 nm was used for the selection of the iridoid glycoside fraction, eliminating the chlorophyll band, which is known to possess an anticancer effect.\textsuperscript{1} Based on TLC results, fractions containing spots with the same retention factor were combined into 1 fraction, concentrated and analyzed by high-performance liquid chromatography-diode array detection-mass spectrometry (HPLC-DAD-MS). Analysis showed that iridoids were present in fractions 9-27. However, for further experiments, the combined iridoid fraction 9-11 was chosen, being the freest of additional components, including chlorophyll.

The HPLC-DAD-MS chromatogram of fraction 9-11 gave evidence of the presence of 2 major and several minor compounds. Both major compounds gave a maximum UV absorption at 238 nm, which was assigned to iridoids. The respective high-resolution mass spectrum (HRMS) of the compound with a retention time of 13.1 minutes (compound 1) showed a pseudomolecular ion peak at \textit{m/z} 583.2012. This dominant peak of fraction 9-11 was identified as sylvestroside III and/or sylvestroside IV, with the molecular formula \textit{C}_{27}\textit{H}_{36}\textit{O}_{14} and an exact mass of 584.2105 Da, based on the fragmentation patterns and comparison with literature data.\textsuperscript{34} In order to confirm the molecular structure of the obtained bis-iridoid glycosides, an MS\textsuperscript{2} analysis was carried out. In the fragmentation spectrum of compound 1, the spectral line at \textit{m/z} 583.2012 is attributable to the deprotonated bis-iridoid glycoside molecule (calculated as \textit{m/z} 583.2027). A fragment at \textit{m/z} 551.1734 (calculated as \textit{m/z} 551.1764) indicated the loss of 32 Da (−O-CH\textsubscript{3}). The loss of glucose and water (−162 Da Glc, −18 Da H\textsubscript{2}O) produced a fragment at \textit{m/z} 403.1385 (calculated as \textit{m/z} 403.1393). The ion represented by the base peak at \textit{m/z} 373.1125 (calculated as \textit{m/z} 373.1134), corresponding to the loss of M−210 Da, further lost the glucosidic part to produce a fragment at \textit{m/z} 193.0499 (calculated as \textit{m/z} 193.0501). It should be noted that the 2 constitutional isomers, sylvestroside III and sylvestroside IV, were likely to produce similar fragmentation spectra in the MS\textsuperscript{2} analysis, and they could not be distinguished under the used experimental conditions.

The second major peak obtained for fraction 9-11 (compound 2) was identified as sylvestroside III−(IV) diethyl acetal, with the molecular formula \textit{C}_{37}\textit{H}_{46}\textit{O}_{13}, and a monoisotopic molecular mass of 658.2837 Da. HRMS analysis revealed the appearance of a deprotonated ion at \textit{m/z} 657.2746 (calculated as \textit{m/z} 657.2759). Fragmentation of the pseudomolecular ion produced 2 parent ions at \textit{m/z} 625.2487 (calculated as \textit{m/z} 625.2493) and \textit{m/z} 447.1853 (calculated as \textit{m/z} 447.1866), which correspond to the loss of the methoxy group (−32 Da) and the iridoid (−120 Da), respectively. The spectral line at \textit{m/z} 267.1224 (calculated as \textit{m/z} 267.1233) belonged to the molecular fragment, which lost iridoid (−120 Da), glucoside (−162 Da), and water (−18 Da). Most likely, this compound has no natural origin and was formed during the acetalization reaction during the treatment of the fraction with ethanol, and, should, therefore, be considered an artifact.

\textit{Cytotoxicity Test}

The cytotoxicity of the iridoid glycoside fraction 9-11 of the alcoholic extract of \textit{D. fullonum} leaves was determined using the propidium iodide (PI) assay, in parallel staining with WST-1. NIH 3T3, MCF7, B16F10, MDB-MD-231, and HeLa cells were treated with the undiluted extract (details on the iridoid fraction preparation are given in Materials and methods) and the fraction diluted 5-fold; the viability parameters were registered after 48 and 72 hours. For quantitation of dead cells the PI assay was used; this did not reveal any statistically significant toxic effect of the extracts on the cells after 48 hours of incubation at either concentration of the fraction. However, the cell incubations with the iridoid glycoside fraction at a concentration of 1:5 for 72 hours gave evidence of a statistically significant increase (3.2 ± 0.2-2.7 ± 0.3-fold over the control, respectively) in the number of dead cells in the MDB-MD-231 cell line, unlike the noncancer cell line NIH 3T3 (Figure 1(A)). The cell treatment with the undiluted extract for 72 hours increased the amount of PI-positive cells in both human breast cancer MCF7 and MDB-MB-231 cell lines, differently from the noncancer cell line NIH 3T3. In the case of NIH 3T3, the
number of PI-permeabilized cells was increased 4.2 ± 0.7-fold over the control, while in the case of MCF7, there was a 5.6 ± 0.2-fold increase and in the case of MDB-MD-231, the increase was 5.8 ± 0.2-fold (Figure 1(B)). The results of the PI assay also demonstrated that the extract exhibited a dose-dependent cytotoxic effect on cell lines MCF 7 and MDB- MB-231, unlike the other cell lines (Figure 1(A) and (B)).

Based on the PI assay results, the cytotoxicity of the undiluted iridoid glycoside fraction was estimated by the WST-1 test only after 72 hours of incubation. The results showed that the fraction induced a statistically significant decrease ($P < .05$) in the viability of MCF7 and MDB-MD-231 cells after 72 hours of incubation, respectively, of 64.0% ± 2.6% and 69.5% ± 7.6% (Figure 2), while the toxicity toward noncancer NIH 3T3 cells was low, 92.8% ± 0.5%, as against the control. The HeLa cells also demonstrated a statistically significant sensitivity to the toxicity of the iridoid glycoside fraction (78.9% ± 4.7%), which can be attributed to the early changes in the metabolic activities of the cells, while the PI assay still did not indicate an increase in the number of dead cells.

Recently, acylated iridoids from Premna odorata (Lamiaceae) were shown to have strong antiproliferative activities against different breast cancer cell lines, including MCF7 and MDB-MB-231, depending on the ERα and c-Met expressions in the cell line.35 In the present study, the researchers demonstrated that nonmodified iridoid glycosides isolated from D. fullonum leaves exerted the selective cytotoxic effect on the same tumor cell lines, while the normal cell line NIH3T3 was much less affected.
Conclusions

In the current study, the bis-iridoid glycoside fraction from the extract of *D. fullonum* leaves was separated and characterized. HPLC-MS analyses demonstrated the fraction to consist mainly of bis-iridoids. The results of the study showed that the iridoids exerted a selective cytotoxic effect on different cancer cells, while no such effect on normal cells was observed. The WST-1 assay established a significant sensitivity of MCF7 and MDB-MD-231 cells toward the iridoid glycosides from *D. fullonum* leaf extracts. The WST-1 assay also gave evidence of a decrease in the mitochondrial function, while the PI detected an increased number of dead cells. Further study will be required to have a more thorough understanding of the anti-breast cancer molecular mechanism of iridoids.

Experimental

**Materials**

All the reagents were of analytical grade and were used as received. Chloroform, *n*-butanol, acetonitrile, formic acid (FA), PI, and phosphate-buffered saline (PBS) were purchased from Sigma & Aldrich (Germany). Methanol and ethanol (EtOH) were purchased from Fluka (Switzerland). Deionized water (Milli Q, Millipore S AS, Molsheim, France) was used for the preparation of all the solutions.

**Extraction**

The leaves of *Dipsacus fullonum*, collected in Saaremaa, Estonia, during June-August 2017, were washed with Milli Q water, dried at room temperature and powdered in a mechanical grinder.

Plant identification was performed by comparison with an authentic specimen *Dipsacus fullonum*, and voucher specimen has been deposited in the Herbarium of Institute of Agricultural and Environmental Sciences of the Estonian University of Life Sciences (TAA), herbarium specimens TAA0153271-TAA0153274.

The conditions and the solvent for extraction were chosen according to a previously published study. Ten grams of air-dried powdered leaves of *D. fullonum* was soaked in 200 mL of 80% methanol in a capped bottle at room temperature for 1 hour and extracted using an ultrasonic bath at 40 °C for 30 minutes. The mixtures were centrifuged at 8000 rpm for 10 minutes and filtered through a 0.45-µm cellulose filter. An aliquot was subjected to a preliminary identification analysis. The main part of the extract was evaporated to dryness in a rotary evaporator, and applied to the column. The column was eluted using a step-gradient elution technique from chloroform to methanol.

The column was developed by adding 10 mL of each eluent while collecting 3 mL fractions; the fractions were evaluated by TLC using a mobile phase of water:methanol:*n*-butanol (1:1:4). A total of 54 fractions were collected. The fractions with the same spots and same retention factors were combined into 1 fraction. The iridoid glycosides fraction was evaporated to dryness. After drying, 5 mg of the solid residue was dissolved in 500 µL of ethanol and subjected to HPLC-MS analysis.

**Chromatographic Analysis**

The chromatographic separations were performed on an Agilent Technologies 6540 UHD Accurate-Mass Q-TOF LC/MS spectrometer equipped with a diode array detector and an AEI-ESI ionization source (Agilent Technologies, Santa Clara, CA, USA). The optimal separations were obtained on an Eclipse Plus C18 column (150 × 2.1 mm, 3.5 µm; Agilent Technologies, USA), with a mobile phase consisting of 0.1% aqueous FA and acetonitrile (containing 0.1% FA). HPLC separation was performed using a continuous gradient elution method. The gradient program was as follows: 0 minutes—0% B, 20 minutes—50% B, 25 minutes—95% B, isocratic for 5 minutes. Then the system was equilibrated for 5 minutes. The detection wavelength, flow rate, column temperature, and injection volume were set at 254 nm, 0.6 mL/min, 20 °C, and 5 µL, respectively. The parameters for MS analysis were set in the negative ion mode, with the spectra obtained over a mass range from 100 to 1000 m/¿. Nitrogen was used for nebulization and for drying the gas, and helium was used as the collision gas. Peak identification was achieved by MS² analysis.

**Cell Cultures**

Murine fibroblast NIH/3T3, mouse melanoma B16F10, HeLa human cervical cancer cells, human breast cancer MCF7 and MDB-MD-231 cell lines were obtained from the American Type Culture Collection. The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% bovine calf serum (Gibco) and 5% penicillin/streptomycin. All the cell lines were incubated at 37°C in a humidified 5% CO₂ and 95% air atmosphere.

**Treatment Procedures and Sample Preparation**

The cells were plated at a density of 2.5 × 10⁵ cells/well (The Countess Automated Cell Counter, Invitrogen) in 96-well plates and incubated overnight. After 24 hours of incubation, 100 µL of either fresh media or fresh media containing the bis-iridoid fraction (1 µL diluted at 1:5 with the EtOH bis-iridoid fraction, or extract/silica gel ratio of 1:20. A dry loading method of the crude extract was used. For this, a certain amount of the crude extract was dissolved in methanol and adsorbed in a small amount of silica gel, dried using a rotary evaporator, and applied to the column. The column was eluted using a step-gradient elution technique from chloroform to methanol.
1 µL of the undiluted bis-iridoid fraction) was added into each well and incubated for a further 48 and 72 hours.

PI Assay
PI is a red-fluorescent DNA-binding dye that is used to detect nonviable cells having disrupted cell membranes, as it cannot cross intact cell membranes. To 100 µL of cell culture, 0.5 mM of PI in PBS was added at 0.5 µL/well and incubated for 10 minutes at 37 °C. The cells, with an addition of 1 µL of 96% EtOH, were used as a negative control and their PI fluorescence was counted as 1. The fluorescence intensity was measured using a TECAN Genios Pro Microplate Reader (excitation 540 nm, emission 612 nm) at 48 and 72 hours after the treatments with the extracts. The normalized results represented the fold increase from the control islets.

Cell Viability Measured by WST-1
The effect of the extracts on the viability of cells was determined using the cell viability assay WST-1 (Roche). WST-1 allowed the colorimetric measurement of cell viabilities, due to the reduction of tetrazolium salts to water-soluble formazan by viable cells. The amount of formed formazan dye correlated with the number of viable cells. The measurements were completed 72 hours after the cell treatments. The experiments, with an addition of 1 µL of 96% EtOH, were used as a negative control. Five microliters per well of the WST-1 reagent was added to 100 µL of the cell culture medium, incubated at 37 °C for 2 hours, after which the absorbance was measured at 450 nm by using a TECAN Genios Pro Microplate Reader.

Statistical Analysis
Statistical analysis was performed using one-way analysis of variance, together with Dunnett’s post hoc multiple comparisons test. The graphs represent data from at least 3 independent experiments, all performed in triplicate, as the mean ± standard deviation. In the cell viability assay, the positive cells were normalized to 100% in the negative control. In the PI assay, the results presented the fold increase from the control islets. A statistical significance of $P < .05$ is represented as *, $P < .01$ as **, and $P < .001$ as ***. Statistical analysis was performed with GraphPad Prism 7.

Declaration of Conflicting Interests
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