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Cytotoxic Deoxypodophyllotoxin Can Be Extracted in High Purity from *Anthriscus sylvestris* Roots by Supercritical Carbon Dioxide

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Key words
Anthriscus sylvestris, Apiaceae, deoxypodophyllotoxin, etoposide, supercritical carbon dioxide extraction

received August 7, 2017
revised November 10, 2017
accepted November 24, 2017

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DOI https://doi.org/10.1055/s-0043-123938
Published online December 18, 2017 | Planta Med 2018; 84: 544–550 © Georg Thieme Verlag KG Stuttgart · New York | ISSN 0032-0943

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ABSTRACT

Deoxypodophyllotoxin is present in the roots of *Anthriscus sylvestris*. This compound is cytotoxic on its own, but it can also be converted into podophyllotoxin, which is in high demand as a precursor for the important anticancer drugs etoposide and teniposide. In this study, deoxypodophyllotoxin is extracted from *A. sylvestris* roots by supercritical carbon dioxide extraction. The process is simple and scalable. The supercritical carbon dioxide method extracts 75–80% of the total deoxypodophyllotoxin content, which is comparable to a single extraction by traditional Soxhlet. However, less polar components are extracted. The activity of the supercritical carbon dioxide extract containing deoxypodophyllotoxin was assessed by demonstrating that the extract arrests A549 and HeLa cells in the G_2/M phase of the cell cycle. We conclude that biologically active deoxypodophyllotoxin can be extracted from *A. sylvestris* by supercritical carbon dioxide extraction. The method is solvent free and more sustainable compared to traditional methods.

ABBREVIATIONS

DPT	deoxypodophyllotoxin
FACS	fluorescence-activated cell sorting
SC-CO_2	supercritical carbon dioxide

Introduction

Podophyllotoxin, which serves as the precursor of several pharmaceutically important antitumor drugs like etoposide and teniposide (▶ **Fig. 1**), is extracted from the roots of *Podophyllum hexandrum*, native to the Himalayan area. Overharvesting has led to the listing of *P. hexandrum* on the Convention on International Trade in Endangered Species of Wild Fauna and Flora list [1]. Therefore, an alternative source for podophyllotoxin has to be found. The lignan DPT can be extracted from the roots of *Anthriscus sylvestris* (L.) Hoffm. (Apiaceae). This common wild plant grows in Europa and temperate Asia, and is considered an invasive species in the Netherlands, Sweden, and Iceland [2–4]. DPT has higher cytotoxicity than podophyllotoxin [5], but it has never been in clinical development. DPT can be converted into epipodophyllotoxin by insertion of a hydroxyl group using cytochrome P450 3A4 produced in *Escherichia coli* [6] or via chemical synthesis [7]. The resulting epipodophyllotoxin can be easily converted into etoposide [6]. Therefore, *A. sylvestris* might become an alternative source to *P. hexandrum* for the production of etoposide.

DPT has been extracted previously by Soxhlet [8], and by sonication [9] for small-scale analysis of the DPT content in *A. sylvestris*. Both methods are strongly dependent on the use of organic solvents, such as methanol. The hazardous nature, high costs, and environmental risks of organic solvent extraction led to the quest for alternative extraction techniques [10]. Green chemistry ap-

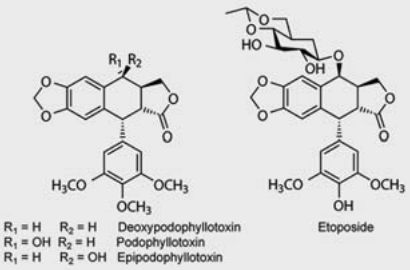
proaches are aimed at the reduction or elimination of organic solvent usage in extraction techniques. A "greener" alternative is supercritical fluid extraction [11]. The most popular fluid for supercritical extraction is carbon dioxide, as it is nonflammable, nontoxic, easily available, and cheap. Furthermore, supercritical conditions are reached at a relatively low pressure (73 bar) and temperature (3 °C) [12, 13]. SC-CO_2 extraction can be used to selectively extract compounds, as the solubility of components can be manipulated by changing the pressure and/or temperature [12]. SC-CO_2 extraction has already been applied for the extraction of lignans from the seeds, fruits, and stems of *Schizandra chinensis* [14, 15]. However, extracting a high yield of lignans from the leaves was only possible by the addition of the cosolvent ethanol [14]. Furthermore, Gupta and coworkers extracted podophyllotoxin from *P. hexandrum* roots using SC-CO_2 extraction and the cosolvents ethyl acetate and methanol [16].

This study focuses on the feasibility of using SC-CO_2, without the addition of organic cosolvents, for the extraction of biologically active DPT from *A. sylvestris* populations in the wild. Furthermore, a novel quick methanol vortex extraction method for analytical determination of the DPT content in *A. sylvestris* roots is provided.

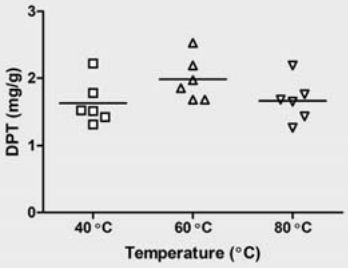
Results and Discussion

An initial experiment showed that DPT can be extracted in the absence of solvents from *A. sylvestris* by SC-CO_2. Subsequently, the parameters for supercritical carbon dioxide as described in the Methods section were altered in a systematic fashion to investigate the most efficient extraction of DPT from *A. sylvestris* roots. A factorial design approach was deployed to find the combination with the highest DPT yield. DPT yields at a pressure of 175 bar were 20% higher than at 100 bar and more reproducible than at 250 bar. Therefore, 175 bar was set as the standard. Extractions for 1 h at 40, 60, and 80 °C yielded comparable amounts of DPT (▶ **Fig. 2**). In total, 1.6 ± 0.3 mg/g DPT was extracted at 40 °C, 2.0 ± 0.3 mg/g at 60 °C, and 1.7 ± 0.3 mg/g at 80 °C. To test for residual DPT in the plant material after extraction at 60 °C, a sequential extraction on the same plant residue was performed by SC-CO_2 (1 h at 60 °C), followed by Soxhlet extraction (2 × 1 h). The SC-CO_2 extraction yielded an additional 0.5 ± 0.1 mg/g and the Soxhlet extraction 0.7 ± 0.06 mg/g (**Fig. 1S**, Supporting Information). Therefore, we calculate that 2.5 ± 0.4 mg/g DPT was extracted at 60 °C after 2 × 1 h extraction at 175 bar by SC-CO_2. Approximately 20–25% of DPT remains in the plant material, which can be extracted by Soxhlet extraction. The presence of DPT in the extracts was confirmed by LC-ESI-MS/MS analysis (fragment ions of *m/z* 231 and *m/z* 187) [17].

The next question was whether the SC-CO_2 extract from *A. sylvestris* was biologically active. DPT binds to tubulin and prevents microtubule assembly resulting in cell cycle arrest at the G_2/M phase, which can be analyzed by FACS analysis of propidium iodide-stained cells [18]. We treated lung epithelial cells (A549) and cervix epithelial cells (HeLa) with SC-CO_2 extract, pure *A. sylvestris* DPT, and etoposide (a DPT-derived drug). Etoposide blocks the cell cycle in the late S or early G2 phase of the cell cycles by inhibition of DNA topoisomerase II [19], and is used, for



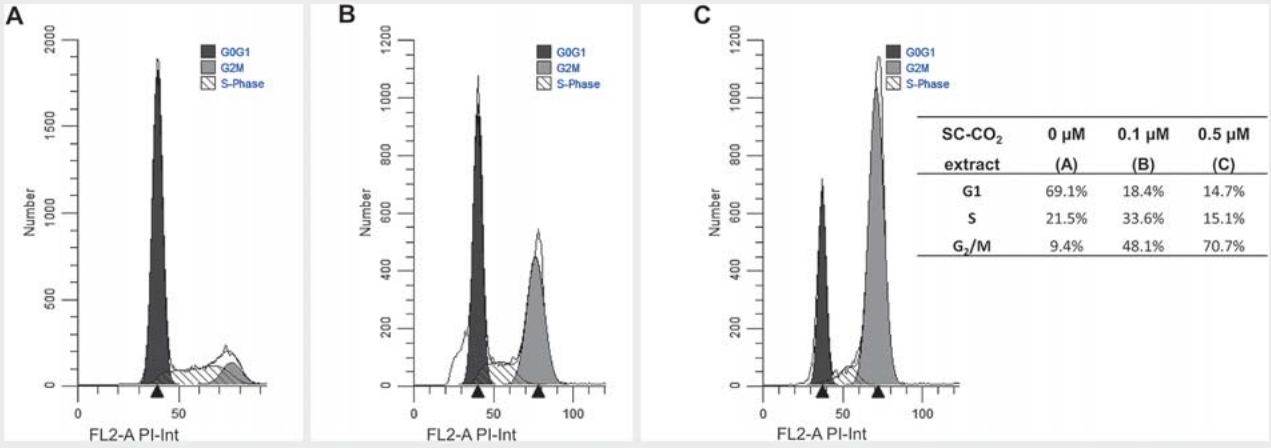
▶ **Fig. 1** Chemical structures of deoxypodophyllotoxin, podophyllotoxin, epipodophyllotoxin, and etoposide.



▶ **Fig. 2** Extraction yields of deoxypodophyllotoxin (DPT) by SC-CO_2 extraction. *A. sylvestris* roots were extracted at 175 bar for 1 h at 40 °C (□), 60 °C (△), or 80 °C (▽). The values have been corrected for dilutions and calculated back to dry weight of the initial plants (n = 6).

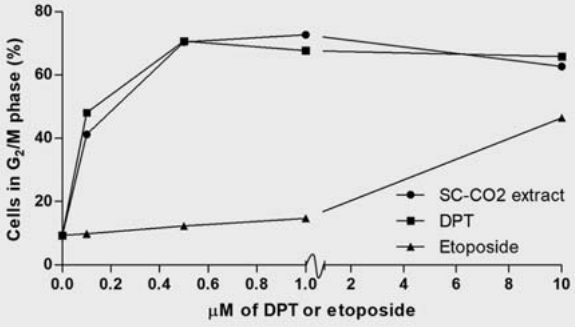
example, in the treatment of small lung cancer [20]. After 24 h treatment, SC-CO_2 extract containing 0.5 µM DPT increased the percentage of cells in the G_2/M phase from 9.4 to 70.4% in A549 cells (▶ **Fig. 3**). This increase is comparable to the one obtained with 0.5 µM pure DPT (70.7%), confirming that the extracted DPT is active (▶ **Fig. 4**). It is noteworthy that the effect of etoposide was less pronounced as the percentage of G_2/M phase cells reached only 46.4% after treatment with the high concentration of 10 µM etoposide (▶ **Fig. 4**). The same trend was observed for HeLa cells (**Fig. 2S**, Supporting Information). These findings show that extract from SC-CO_2 extraction is capable of arresting cells in the G_2/M phase of the cell cycle in a dose-dependent manner that correlates well with the dose-response curve of pure DPT. This suggests that DPT accounts for the cytotoxic activity of the SC-CO_2 extract, which is in concert with the findings using methanolic extracts of *A. sylvestris* [18]. The high activity on cell cycle arrest by pure DPT is in accordance to literature values [18, 21]. Interestingly, at similar concentrations, the clinically used etoposide was much less potent in obtaining arrest in the G_2/M phase. The difference in the action mechanism, topoisomerase inhibition for etoposide versus tubulin destabilization for DPT, might be responsible for this [22].

In order to assess the new extraction method, we have compared (i) the solvent-free SC-CO_2 extraction method to (ii) the

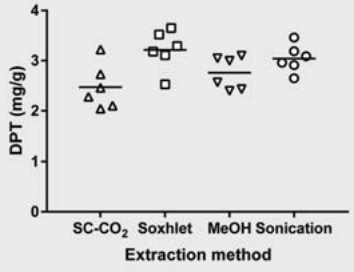


▶ **Fig. 3** Cell cycle arrest of A549 cells treated with SC-CO_2 extract. A549 cells were treated with SC-CO_2 extract containing 0 (**A**), 0.1 (**B**), or 0.5 µM (**C**) deoxypodophyllotoxin for 24 h. FACS was used as the method of analysis.

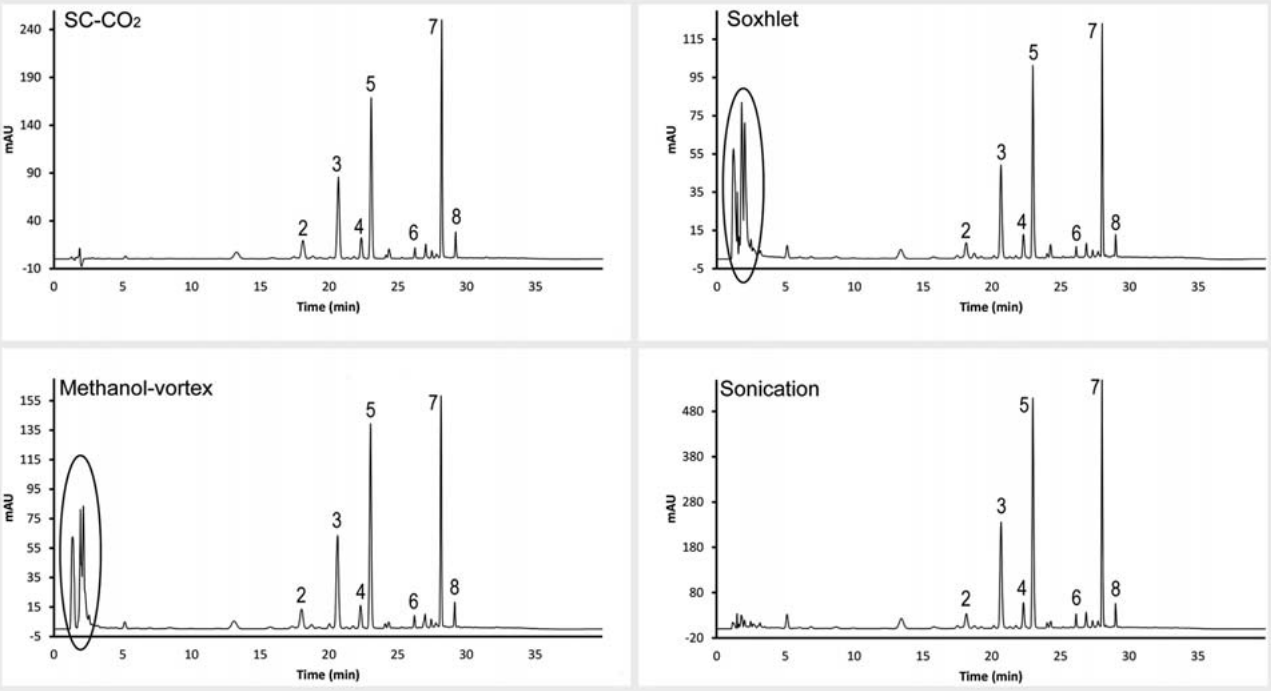
Soxhlet, (iii) a methanol vortex extraction, and (iv) a sonication method. The DPT absolute yields for the SC-CO_2 method were compared to the other methods (▶ **Fig. 5**). As mentioned earlier, the yield of SC-CO_2 extraction at 175 bar after 2 × 1 h is 2.5 ± 0.4 mg/g (i). DPT extracted by Soxhlet extraction (ii) yielded 3.2 ± 0.5 mg/g after two rounds of extraction. The new analytical methanol vortex extraction method (iii) after extraction three times gave a yield of 2.8 ± 0.3 mg/g. Extraction by sonication (iv) yielded 3.1 ± 0.4 mg/g DPT. An additional round of extraction did not result in higher yields for any of the methods. Significantly more DPT was extracted by Soxhlet (p value = 0.012) and sonication (p value = 0.023) than by SC-CO_2 extraction. The yield of the methanol vortex extraction was not significantly different from the yields obtained with the other methods. Apart from absolute yield, we also looked at the cleanness of the HPLC profiles. Additional polar plant components were observed with the Soxhlet (ii) and methanol vortex methods (iii) (encircled peaks in ▶ **Fig. 6**). These peaks were absent from the HPLC chromatogram of the SC-CO_2 (i) and sonication (iv) method. This study shows that DPT can be extracted from *A. sylvestris* by SC-CO_2 extraction in a reasonable yield, as around 75–80% of the DPT was recovered. Furthermore, the HPLC profile of the SC-CO_2 extraction is cleaner than that of the Soxhlet extraction. This is caused by the absence of polar components, which will not be extracted by SC-CO_2 extraction and therefore remain in the plant residue. In contrast, these polar components are extracted in the Soxhlet and methanol vortex methods, as observed in the HPLC chromatograms where they are eluted with the front of the solvent peak. This suggests that the SC-CO_2 (i) and sonication (iv) methods could be more selective. Furthermore, the removal of CO_2 in a gaseous state reduces the volume in further downstream processes. LC-ESI-MS/MS analysis confirmed the presence of six lignans in all of the extracts: isopicropodophyllone (**1**), podophyllotoxone (**2**), DPT (**3**), yatein (**4**), anhydropodorhizol (**5**), and angeloyl podophyllotoxin (**6**) (▶ **Table 1**, **Fig. 6**, and **Fig. 3S**, Supporting Information). Additionally, the compounds anthriscrusin (**7**), and



▶ **Fig. 4** Cell cycle arrest of A549 cells in the G_2/M phase after 24 h treatment. Cells were treated with SC-CO_2 extract containing 0, 0.1, 0.5, 1, or 10 µM deoxypodophyllotoxin (DPT), pure DPT, or etoposide (n = 1).



▶ **Fig. 5** Comparison of deoxypodophyllotoxin (DPT) extraction yields by various extraction methods. The extraction yields of SC-CO_2 (△), Soxhlet (□), methanol vortex (MeOH, ▽), and sonication (○) were compared. The values have been corrected for dilutions and calculated back to dry weight of the initial plants (n = 6). P value < 0.05, Student's t-test.



▶ **Fig. 6** HPLC profiles of *A. sylvestris* root extracted by various extraction methods. The four extraction methods were SC-CO_2, Soxhlet, methanol vortex, and sonication (top to bottom). HPLC chromatograms were analyzed at 289 nm. The components were identified by LC-ESI-MS/MS (▶ **Table 1**). The encircled peaks represent polar components that were only extracted by Soxhlet and the methanol vortex method.

▶ **Table 1** Overview of components found in *A. sylvestris* roots extracts.

No	Compound	MW	Quasi-molecular ions $[M + NH_4]^+$	Fragment ions
1	Isopicropodophyllone	412	430	245, 201
2	Podophyllotoxone	412	430	245, 201
3	Deoxypodophyllotoxin	398	416	231, 187
4	Yatein	400	418	223, 181
5	Anhydropodorhizol	398	416	231, 135
6	Angeloyl podophyllotoxin	496	514	397, 313, 229
7	Anthriscrusin	388	406	191
8	2-methyl-4-[[(2Z)-2-methyl-1-oxo-2-buten-1-yl]oxy]-,(2E)-3-(7-methoxy-1,3-benzodioxol-5-yl)-2-propen-1yl ester, 2(Z)-2-butenoic acid	388	406	191

Compounds **1**–**5**, **7**, and **8** were identified by Multiple Reaction Monitoring based on the data of Hendrawati and coworkers [2]. Compound **6** was identified by Product Ion Scan and compared to the data of Koulman and coworkers [3].

2-methyl-4-[[(2Z)-2-methyl-1-oxo-2-buten-1-yl]oxy]-,(2E)-3-(7-methoxy-1,3-benzodioxol-5-yl)-2-propen-1yl ester, 2(Z)-2-butenoic acid (**8**) were detected (▶ **Table 1**, **Fig. 6**, and **Fig. 3S**, Supporting Information). Identification of the peaks was based on the data of Hendrawati et al. and Koulman et al. [9, 17]. In all four extracts, the fingerprint of these peaks was similar, indicating that all extraction methods are equally capable of extracting lignans present in *A. sylvestris* roots. The lignans found in this study are structurally related to DPT. The main lignan peaks found were DPT (**3**) and anhydropodorhizol (**5**) (peak area, ▶ **Fig. 6**). Anhydropodorhizol is structurally linked to yatein, which is a precursor of DPT [23, 24]. Therefore, it could be of interest to increase the DPT yields by pathway engineering aimed at converting anhydropodorhizol to DPT [25].

DPT is a precursor of podophyllotoxin, which can be converted to the pharmaceutically important anticancer drugs etoposide and teniposide. Since the natural source of podophyllotoxin, *P. hexandrum*, is endangered in its native habitat, we were interested in the extraction of DPT from *A. sylvestris*. The SC-CO_2 extraction method has been used to extract lignans from various plant material and components from root material, but has not been described yet for the extraction of DPT from *A. sylvestris*. Furthermore, DPT has not been extracted before from a plant without the addition of a cosolvent. We showed that low volume and DPT-enriched *A. sylvestris* extracts can be obtained by SC-CO_2 extraction. The SC-CO_2 method can be scaled up for industrial application, which has already been done for the decaffeination of coffee and tea [26]. Therefore, the SC-CO_2 method has the potential to be used in the future for large-scale extraction of DPT from *A. sylvestris*. A quick methanol vortex extraction method was developed, which can be used for quantification of the DPT content in *A. sylvestris* roots. This can be convenient for plant breeding programs of *A. sylvestris* aimed at higher DPT production yields. Taken together, this research underscores the importance of *A. sylvestris* as a novel source for anticancer drugs. Although, further research is necessary to determine if *A. sylvestris* can become a cash crop for farmers.

Materials and Methods

Plant material

Roots of *A. sylvestris* were collected in May 2013 from flowering populations at various locations in the province of Groningen, The Netherlands. The plants were identified by Christel Seegers using the Dutch flora book [27]. Voucher specimens have been deposited in the collection of the University of Groningen; Asylv2013. The roots were collected, rinsed with tap water, and dried overnight at 30 °C. All roots were pooled, cut into pieces, ground, and sieved (1–2.8 mm).

Chemicals

Technical methanol (98.5 %, v/v) and acetonitrile (99.8 %, v/v) were purchased from VWR. Ammonium formate (> 97 %, v/v), propidium iodide (> 94 %, v/v), and the reference compound etoposide (≥ 98 %) were purchased from Sigma-Aldrich. Other chemicals used were methanol absolute AR (99.8 %, v/v; Biosolve), formic acid (98–100 %; Merck), carbon dioxide (99.7 %, v/v; Linde), triton X-100 (Fluka Biochemica), and RNAse A (Qiagen). The cell lines A549 and HeLa were obtained from ATCC. Reference compound DPT [> 98 % pure, ^{1}H NMR ($CDCl_3$) and HPLC-ESI/MS, **Fig. 4S**, Supporting Information] for HPLC and LC-ESI-MS/MS analysis was isolated from *A. sylvestris* at the Department of Chemical and Pharmaceutical Biology, Groningen, The Netherlands by the method of van Uden [8]. DPT [98 % pure, ^{1}H NMR ($CDCl_3$) and HPLC-ESI-MS/MS, **Fig. 5S**, Supporting Information] for FACS analysis was purchased from Toronto Research Chemicals.

Extraction of deoxypodophyllotoxin from plant roots

SC-CO_2 extraction as a "green process" was compared with Soxhlet, methanol-vortex, and sonication for extraction of DPT from *A. sylvestris* roots. Root fragments varying from 1 to 2.8 mm were used for the extractions.

Supercritical carbon dioxide extraction

The SC-CO_2 extraction method was designed with a future large-scale extraction of DPT in mind. The high-pressure setup consists of a stirred batch reactor (Parr Instrument, 100 mL), an electrical heating element with temperature controller, a high-pressure pump unit, and a carbon dioxide feeding bottle (**Fig. 6S**, Supporting Information). The carbon dioxide was supplied to the reactor using a membrane pump (Lewa, capacity 60 kg/hr, maximum pressure 35 MPa). To prevent cavitation in the pump, the carbon dioxide was first cooled to 0 °C in a heat exchanger (Huber). After pressurizing, a second heat exchanger with hot oil was used to heat the carbon dioxide to the desired temperature [28].

For extraction, a spinning basket was filled with 1 g of plant material and placed on the stirrer in the batch reactor. A heat exchanger was placed around the reactor and the reactor was filled with CO_2 until the desired pressure was achieved (between 15 and 42 g of CO_2). The plant material was extracted in a static extraction system for 1 h at 90 rpm. A factorial design was used to establish the most critical parameters: pressure (100, 175, and 250 bar) and temperature (40, 60 and 80 °C). After the extraction, the reactor was cooled down to 30 °C and depressurized. The residue in the reactor was dissolved in methanol and transferred to a 25-mL volumetric flask. The amount of DPT was determined by HPLC using a calibration curve. Samples were stored at 4 °C before analysis.

Soxhlet extraction

In the literature, up to now, the report on DPT extraction was by the traditional Soxhlet method [8]. We adjusted the protocol to a small-scale extraction method performed in a Tecator Soxtec System HT2 comprising two 1045 extraction units connected to an oil heating device (1046 service unit; Gemini). One gram of plant material was transferred to a cellulose thimble (FOSS Benelux BV) and extracted three times (80 mL methanol) for 1 h. After every extraction step, the thimble was rinsed with the solvent three times before the beaker was refilled with fresh solvent. The first two extractions were pooled and concentrated, and the volume was adjusted to 100 mL in a volumetric flask. The volume of the third extraction was concentrated and adjusted to 20 mL. The amount of DPT was determined by HPLC analysis. Samples were stored at 4 °C before analysis.

Methanol vortex extraction

For analytic purposes, a quick methanol vortex extraction method was designed for extraction of DPT from *A. sylvestris* roots. Ten mL of methanol were added to 1 g of plant material. The sample was vortexed for 30 s on a Heidolph Reax top, at 2500 rpm (Heidolph), followed by 10 min of centrifugation (2900 *g* and 4 °C) to separate the supernatant from the solid fraction. This extraction was repeated four times. The first three supernatants were pooled and the volume was adjusted to 50 mL in volumetric flasks. The fourth supernatant was kept separate and the volume was adjusted to 25 mL in a volumetric flask. The DPT concentration was determined by HPLC analysis. Samples were stored at 4 °C before analysis.

Sonication

DPT has been extracted by sonication as described previously [9]. Briefly, 100 mg of dried plant material were weighed into a Sovirel tube. The sample was sonicated for 1 h in a Brandson 5210 ultrasonic bath (Boom B. V.) after the addition of 2 mL 80 % of methanol. Subsequently, 4 mL of dichloromethane and 4 mL of water were added. The mixture was vortexed and centrifuged (1000 *g*, 5 min). The organic layer was transferred to Eppendorf tubes and dried overnight in the fume hood and dissolved in 2 mL of methanol (volumetric flask). The amount of DPT was determined by HPLC. Samples were stored at 4 °C before analysis.

Assessment of deoxypodophyllotoxin amount by HPLC

The amount of DPT was analyzed by HPLC as previously described [29], with some modifications. A Shimadzu-VP system was used, consisting of an LC-10AT pump, SIL-20A autosampler, and diode array detector SPD-M10A. A Zorbax Eclipse XDB-C18 column (4.6 × 150 mm; 5 µm; Agilent) and an Eclipse XDB-C18 guard column containing cartridges (4.6 id. × 12.5 mm, 5 µm; Agilent) were used for the analysis. The mobile phase consisted of water/acetonitrile (95 : 5) (A) and acetonitrile/water (95 : 5) (B), both supplemented with 0.1 % formic acid and 2 mM ammonium formate. The elution flow rate was 1 mL/min and the column temperature was held constant at 25 °C. The injection volume for the standard and extracts was 20 µL. A gradient program was performed that consisted of gradient buffer A–B: 10 min 70 : 30 (v/v) isocratic; gradient 8 min 50 : 50 (v/v); gradient 7 min 10 : 90 (v/v); 5 min 10 : 90 (v/v) isocratic; gradient 5 min 70 : 30 (v/v); 5 min 70 : 30 (v/v) isocratic. The HPLC method was able to separate DPT from the other compounds. The extracts were diluted in methanol (see Extraction section) to obtain DPT concentrations within the range of the calibration curve. The procedure was validated according to ICH guidelines [30]. Evaluation of linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy are presented in **Table 1S**, Supporting Information.

Identification of deoxypodophyllotoxin by LC-ESI-MS/MS

The presence of DPT and related lignans in the extracts was confirmed by LC-ESI-MS/MS. The analysis was performed using a Shimadzu LC system consisting of 2 LC-20AD gradient pumps and a SIL-20AC autosampler. The LC system was coupled to an API 3000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) via a TurboIonSpray source. Data were collected and analyzed by Analyst 1.5.2 acquisition software (Applied Biosystems/MDS Sciex). An Alltima C18 (Grace Davision) narrow-bore guard column (2.1 × 150 mm, 5 µm) was used. Buffers and the gradient program were the same as for HPLC analysis. The ionization was performed by electrospray in the positive mode [$(M + NH_4)^+$ adduct ions]. The source temperature was set to 450 °C. The instrument was operated with an ionspray voltage of 5.2 kV. Nitrogen was used for both the curtain gas and nebulizing gas. Full scan mass spectra were acquired at a scan rate of 1 scan/4 sec with a scan range of 100–1400 amu and a step size of 0.1 amu.

Analysis of cell cycle by flow cytometry

Cell cycle arrest was studied in A549 and HeLa cells by FACS. A549 cells were cultivated in DMEM/F12 media and HeLa cells in DMEM media. Both media were supplemented with 10 % fetal calf serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. The cell lines were cultivated in a humidified incubator at 37 °C containing 5 % CO_2. One million cells were seeded in 6-well plates and treated with different concentrations of SC-CO_2 extract, pure DPT, or etoposide (0, 0.1, 0.5, 1, and 10 µM) for 24 h. Cells were fixated in 70 % ice-cold ethanol and stained in 300 µL propidium iodide solution [1 % (v/v) Triton X-100, 200 µg/mL RNase A, and 20 µg/mL propidium iodide]. The DNA contents of 20 000 events were measured by flow cytometer (Becton Dickinson). Histograms were analyzed using Modfit LT 4.1 software.

Statistics

Statistical analysis was performed with SPSS 23 software. Comparative statistical analysis of the groups was performed using Student's t-test (n = 6). The lines in ▶ **Figs. 2** and **5** represent the mean. The values in the text are reported as the mean ± SD. P values < 0.05 were considered significant.

Supporting information

HPLC validation, HPLC profile Soxhlet extract of SC-CO_2 extracted roots, cell cycle arrest of HeLa cells, chemical structure of compounds **1**–**8**, and the experimental setup are available as Supporting Information.

Acknowledgements

The authors thank H. J. Heeres and M. H. de Vries of the Department of Engineering and Technology of the University of Groningen for the usage of the supercritical carbon dioxide equipment. The authors thank C. M. Jeronimus-Stratingh of the Mass Spectrometry Core Facility of the University of Groningen for the LC-ESI-MS/MS analysis. This work was supported by EU regional funding and The PhytoSana project in the INTERREG IV A Deutschland-Nederland program: 34- INTERREG IV A I-1-01=193.

Conflict of Interest

The authors declare no conflict of interest.

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