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Podophyllotoxin and Deoxypodophyllotoxin in *Juniperus bermudiana* and 12 Other *Juniperus* Species: Optimization of Extraction, Method Validation, and Quantification

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Supporting Information

ABSTRACT: The lignans podophyllotoxin and deoxypodophyllotoxin are secondary metabolites with potent pharmaceutical applications in cancer therapy. However, the supply of podophyllotoxin from its current natural source, *Podophyllum hexandrum*, is becoming increasingly problematic, and alternative sources are therefore urgently needed. So far, podophyllotoxin and deoxypodophyllotoxin have been found in some *Juniperus* species, although at low levels in most cases. Moreover, extraction protocols deserve optimization. This study aimed at developing and validating an efficient extraction protocol of podophyllotoxin and deoxypodophyllotoxin from *Juniperus* species and applying it to 13 *Juniperus* species, among which some had never been previously analyzed. *Juniperus bermudiana* was used for the development and validation of an extraction protocol for podophyllotoxin and deoxypodophyllotoxin allowing extraction yields of up to 22.6 mg/g DW of podophyllotoxin and 4.4 mg/g DW deoxypodophyllotoxin, the highest values found in leaf extract of *Juniperus*. The optimized extraction protocol and HPLC separation from DAD or MS detections were established and validated to investigate podophyllotoxin and deoxypodophyllotoxin contents in aerial parts of 12 other *Juniperus* species. This allowed either higher yields to be obtained in some species reported to contain these two compounds or the occurrence of these compounds in some other species to be reported for the first time. This efficient protocol allows effective extraction of podophyllotoxin and deoxypodophyllotoxin from aerial parts of *Juniperus* species, which could therefore constitute interesting alternative sources of these valuable metabolites.

KEYWORDS: podophyllotoxin, deoxypodophyllotoxin, extraction, *Juniperus*, method validation

INTRODUCTION

Podophyllotoxin, **1** (Figure 1), is a well-known plant secondary metabolite that possesses a pronounced cytotoxic effect on cancer cell lines by inhibition of microtubule formation and serves as a unique starting compound for the hemisynthesis of anticancer drugs that are known to inhibit topoisomerase II such as etoposide (VP16), teniposide (VM26), or etopophos.[1] These synthetic derivatives are widely used to cure lung, testicular, pancreatic, and stomach cancers as well as myeloid leukemias.[2,3] Antiviral activities have also been assigned to podophyllotoxin due to its ability to inhibit the replication of *Herpes simplex* type I virus,[4] and some of its derivatives possess pronounced anti-HIV properties.[5]

However, the availability of podophyllotoxin is becoming increasingly limited due to the fact that it is traditionally extracted from rhizomes of *Podophyllum hexandrum*, a perennial herb found in the Himalayas. It contains a high amount of podophyllotoxin (around 4% of DW). This species is endangered by overcollection, which exceeds its regeneration capacity, and a lack of cultivation.[1] As a consequence, *P. hexandrum* is now listed on Appendix II of The Convention on International Trading of Endangered Species (CITES). A total synthetic approach to obtaining podophyllotoxin is be a workable solution because it is still commercially too expensive due to its complex structure.[6] This supply issue triggered an active search for alternative natural sources, and extraction of podophyllotoxin from biological material is an attractive approach for pharmaceutical industries.

The way in which the supply issue of paclitaxel (taxol), another potent anticancer drug, was solved can be a powerful source of

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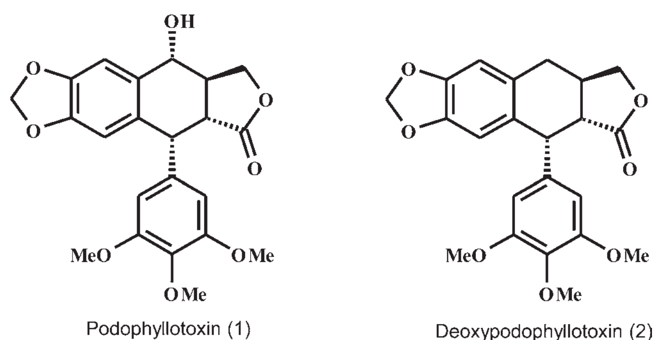

Figure 1. Chemical structures of podophyllotoxin (**1**) and deoxypodophyllotoxin (**2**).

inspiration. Initially, paclitaxel was isolated from the bark of *Taxus brevifolia*, destroying a resource that is extremely slow to regenerate, but it is now obtained by semisynthetic conversion of relatively abundant precursors (baccatins) accumulated in the leaves of various *Taxus* species, which constitute a constantly renewable and nondestructive natural source.[7] Consequently, a cultivated or ornamental plant with high podophyllotoxin or derivatives content in its aerial parts could be an ideal alternative source. In the same genus, *Podophyllum peltatum* has been investigated as an alternative source, but its podophyllotoxin accumulation is low and its cultivation is problematic.[6] Conifer species belonging to *Podocarpus*, *Callitris*, or *Juniperus* genera are known to contain podophyllotoxin and other 2,7′-cyclolignans such as deoxypodophyllotoxin, **2** (Figure 1).[8]

Deoxypodophyllotoxin could be an interesting alternative to podophyllotoxin: it has demonstrated a wide range of relevant properties against a number of cancer cell lines,[9−13] and its stereoselective hydroxylation into epipodophyllotoxin, by recombinant human cytochrome P450 3A4 expressed in *Escherichia coli*, was reported and offered a promising alternative strategy for the large-scale production of this valuable precursor in the pharmaceutical industry.[14,15] The occurrence of podophyllotoxin and deoxypodophyllotoxin in some *Juniperus* species has been described since 1953 by Hartwell et al.,[16] although in most cases at low levels (Table 1). This could be due to the fact that few of these studies focused on the optimization of the extraction process. Aerial green parts of some *Juniperus* species are largely available either as raw material already commonly used for the extraction of essential oil (e.g., *Juniperus communis*, aka common juniper[17]) or as a byproduct of the wood industry (*Juniperus virginiana*, aka Eastern red cedar[18]).

The aim of the present study was to develop and validate an efficient extraction and quantification protocol of podophyllotoxin and deoxypodophyllotoxin from the leaves of *J. bermudiana* and its application to other *Juniperus* species. For this purpose, 13 species were used in this study, most of them belonging to the "Sabina section", characterized by a cypress-like aspect due to scale-like leaves (*J. bermudiana*, *J. deppeana*, *J. flaccida*, *J. martinezii*, *J.* × *media*, *J. phoenicea*, *J. procumbens*, *J. sabina*, *J. scopulorum*, *J. squamata*, and *J. virginiana*). Two other species belong to the "Juniperus section" (*J. communis* and *J. conferta* (also sometimes referred to as *J. rigida conferta*)). Some of the species chosen, such as *J. conferta*, *J. deppeana*, or *J. flaccida*, have not been assayed yet for the presence of podophyllotoxin or related compounds, and for some other species such as *J. bermudiana* or *J. phoenicea* the presence of such compounds was previously reported but never quantitated (Table 1).

Table 1. Published Results Concerning the Occurrence of Podophyllotoxin and Deoxypodophyllotoxin in *Juniperus* Species

Juniperus species	podophyllotoxin content (mg/g DW)	deoxyodophyllotoxin content (mg/g DW)	ref
J. bermudiana	/[a]	nq[b,c]	28
J. blaaws	/	0.005[d]	24
J. chinensis	0.025[e]	/	23
J. communis	0.02[d]	2.1[d]	24
J. × *media* (Pfitzeriana)	0.97[d]	3.3[d]	24
J. phoenicea	/	nq[c]	29
J. procumbens (Tremonia)	/	0.02[d]	24
J. recurva	0.06[d]	1.63[d]	24
J. sabina	1.4[c]	/	16
	/	1.3[f]	21
J. scopulorum	1.7[c]	/	16
J. squamata	0.005[d]	1.67[d]	24
J. taxifolia	/	nq[c]	12
J. virginiana	1[c]	/	16
	4.8[c]	/	25
	1.56[c]	/	26
	3.6[c]	/	27

[a] /, not reported. [b] nq, not quantified. [c] From leaves. [d] Aerial part (stem and leaves). [e] From callus. [f] From fruit.

MATERIALS AND METHODS

Plant Materials. The 13 *Juniperus* species used is the present study were 11 *Juniperus* species with cypress scale-like leaves belonging to section Sabina and two species with needle-like leaves belonging to section Juniperus (*J. communis* and *J. conferta*). Most of them were harvested in botanical gardens: *J. virginiana*, *J. scopulorum*, *J. phoenicea*, *J. bermudiana*, and *J. deppeana* in the INRA Arboretum of Villa Thuret (Juan-les-Pins, France); *J. sabina* in Parc Floral (Orléans, France); and *J. flaccida* and *J. martinezii* in the CUCBA Botanical Garden (Guadalaraja, Mexico). The other species, that is, *J.* × *media*, *J. procumbens*, *J. squamata*, *J. communis*, and *J. conferta*, were cultivars sold for landscaping purchased from a garden center (Chartres, France).

Chemicals. All solvents for extraction and HPLC analysis were of analytical grade or higher available purity and were purchased from Fischer Scientific (Illkirch, France). Deionized water was purified by a Milli-Q water purification system from Millipore (Molsheim, France). All solutions prepared for HPLC were filtered through 0.45 μm nylon syringe membranes prior to use. Podophyllotoxin standard was purchased from Chromadex (Molsheim, France), and deoxypodophyllotoxin was purified from *Anthriscus sylvestris* as described previously.[19]

Extraction Method. *Juniperus* samples were systematically lyophilized before grinding. Typically, 25 mg of DW leaves of *Juniperus* were extracted in 2.5 mL of various concentrations of different solvents (methanol, ethanol, acetone, chloroform, or petroleum ether) and ground using a blender (Ultraturrax, T25 basic) for 3 min at 19000 rpm until a fine suspension was obtained. This mixture was extracted for a duration ranging from 0 to 72 h at a temperature ranging from 4 to 75 °C under stirring in a water bath. The solid residue was centrifuged for 15 min at 3000 rpm, and the supernatant was filtered through 0.45 μm nylon syringe membranes prior to HPLC injection.

When performed, alkaline hydrolysis and enzymatic digestion were accomplished as follows: the supernatant of extraction was mixed with NaOH (0.2 N final concentration) for 6 h at 40 °C, followed by neutralization with acetic acid and filtration through 0.45 μm nylon

syringe membranes prior to HPLC injection; β-glucosidase- or cellulase-assisted release was accomplished using a 2 units/mL enzyme preparation (β-glucosidase (EC 3.2.1.21) from almonds (G0395) (Sigma, Saint Quentin Fallavier, France); cellulase R10 (EC 3.2.1.4) from *Trichoderma reesei* (Merck, Fontenay Sous Bois, France)) in pH 4.8 citrate−phosphate buffer at 40 °C for a duration of 16 h and followed by evaporation leading to complete dryness, resuspension into methanol, and filtration through 0.45 μm nylon syringe membranes prior to HPLC injection.

Proposed Method. *Juniperus* samples were lyophilized before grinding. Typically 25 mg of DW leaves of *Juniperus* was extracted in 2.5 mL of 100% methanol and ground using a blender (Ultraturrax, T25 basic) for 3 min at 19000 rpm until a fine suspension was obtained. This mixture was extracted for 5 h at 25 °C under stirring in a water bath. The solid residue was centrifuged for 15 min at 3000 rpm, and the supernatant was filtered through 0.45 μm nylon syringe membranes prior to HPLC injection.

Chromatographic Analysis of Podophyllotoxin and Deoxypodophyllotoxin. The determination of podophyllotoxin and deoxypodophyllotoxin was carried out on a Varian liquid chromatographic system including a Varian Prostar 230 pump, a Metachem Degasit, a Varian Prostar 410 autosampler, and a Varian Prostar 335 Photodiode array detector (PAD) and controlled by Galaxie version 1.9.3.2 software. The separation was performed at 35 °C. The column used was a 250 mm × 4.0 mm i.d., 5 μm, Purospher RP-18 (Merck).

The mobile phase consisted of 0.2% acetic acid in water (solvent A) and methanol (solvent B). The composition of the mobile phase varied during runs according to a nonlinear gradient at a flow rate of 0.8 mL/min as follows: from 0 to 40 min of A/B, 90:10 (v/v) to 30:70 (v/v); from 40 to 50 min of A/B, 30:70 (v/v) to 0:100 (v/v), and A/B, 0:100 (v/v) from 50 to 60 min. Detection was performed at 280 nm. Podophyllotoxin and deoxypodophyllotoxin were initially identified by comparison of their retention times and their UV spectra to those of standard. Quantification was performed using calibration curves of podophyllotoxin and deoxypodophyllotoxin.

Mass Spectrometry Analysis. The assignments of podophyllotoxin and deoxypodophyllotoxin were facilitated by electrospray tandem mass spectrometry (HPLC-ESI-MS) using a Micromass ZQ Waters mass spectrometry system coupled to a single quadrupole. HPLC separation was carried out as described above. Detection was performed in ES^+ splitless mode: capillary voltage, 300/3.5 kV; cone tension, 35 V; source temperature, 130 °C; desolvation temperature, 350 °C; desolvation gas flow, 450 L/h; and cone flow, 50 L/h. Mass spectrometry results were analyzed using Empower2 software.

Statistical Treatment of Data. All data presented in this study are the mean and standard deviation of at least three independent replicates. Comparative statistical analyses of groups were performed using Student's *t* test or one-way analysis of variance according to the data. All statistical tests were considered to be significant at $P < 0.05$.

■ RESULTS AND DISCUSSION

HPLC Separation Conditions and Preliminary Works. First experiments were conducted to optimize the chromatographic conditions in terms of resolution and peak separation. Best results were obtained using a Purospher RP-18 column eluted with a nonlinear gradient of acetic acid acidified water and methanol. According to the maximum absorption wavelengths of podophyllotoxin and deoxypodophyllotoxin, the detector wavelength was set at 280 nm. On the basis of the above optimizations, the obtained chromatograms presented no interference, and each peak had good resolution (Figure 2).

Preliminary works were also conducted using a direct extraction procedure using the following protocol to determine the better *Juniperus* species to be used for extraction condition optimization. Briefly, 25 mg of *Juniperus* leaves was ground for 3 min with a blender (Ultraturrax) in 2.5 mL of methanol (100%), a procedure typically used for lignan and polyphenol extractions.[20] Thirteen *Juniperus* species (*J. bermudiana, J. communis, J. conferta, J. deppeana, J. flaccida, J. martinezii, J. × media, J. phoenicea, J. procumbens, J. sabina, J. scopulorum, J. squamata, J. virginiana*) were assayed. The best yield of podophyllotoxin was obtained with *J. bermudiana* at 4.1 mg/g DW. Under these extraction conditions, whatever the *Juniperus* species assayed, deoxypodophyllotoxin was not detected. *J. bermudiana* was chosen to design an optimized extraction protocol of podophyllotoxin from *Juniperus* leaves.

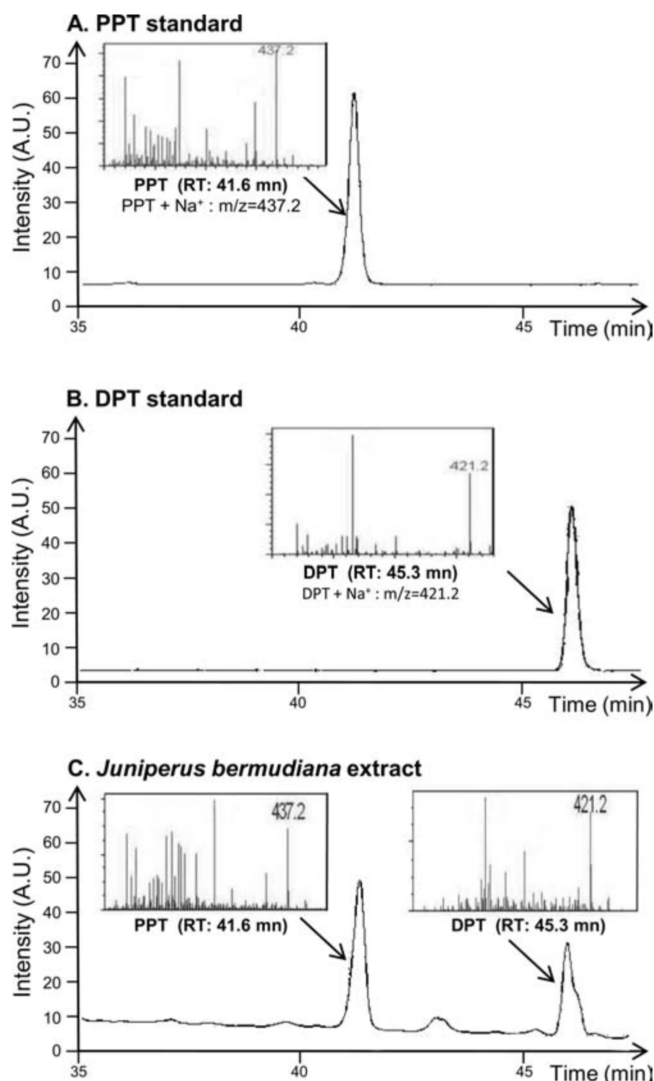

Figure 2. Typical HPLC chromatograms (at 280 nm) and MS spectra of podophyllotoxin (PPT) standard (A), deoxypodophyllotoxin (DPT) standard (B), and methanol extract of leaves from *Juniperus bermudiana* (C).

Optimization of Podophyllotoxin and Deoxypodophyllotoxin Extraction from *J. bermudiana*. Previously published works had shown that acetone, chloroform, methanol, and petroleum ether could be suitable solvents for the extraction of deoxypodophyllotoxin from *J. sabina*.[21] It was decided to assay these solvents for a direct extraction of podophyllotoxin from leaves of *J. bermudiana*. Best results were obtained using 100%

methanol with a podophyllotoxin content of 4.1 mg/g DW and acetone with 3.2 mg/g DW (Figure 3A). Lower yields were obtained with chloroform and ethanol (1.9 and 1.6 mg/g DW podophyllotoxin, respectively; Figure 3A). Petroleum ether led to poor extraction yield (0.5 mg/g DW podophyllotoxin). These results clearly demonstrated that among all tested solvents methanol was the more efficient for the extraction of podophyllotoxin from the leaves of *J. bermudiana*.

The extraction capacities of different methanol/water ratios were evaluated, and 100% methanol was found to be the optimal concentration (Figure 3B). Extending the extraction duration to 5 h with 100% methanol resulted in a dramatic increase in the podophyllotoxin extraction yield with a content of 22.6 mg/g DW (Figure 4). This podophyllotoxin extraction yield is by far the best ever obtained in a *Juniperus* species (Table 1). Extending the extraction duration also made it possible to extract and quantify deoxypodophyllotoxin with a higher yield of 4.4 mg/g DW after 5 h of extraction (Figure 4), which also constitutes the best yield of deoxypodophyllotoxin obtained from a *Juniperus* species (Table 1). Extended incubation duration beyond 5 h resulted in major decreases of podophyllotoxin and deoxypodophyllotoxin contents (Figure 4), indicating that both are susceptible to hydrolysis or modifications in methanol upon extending extraction. This was particularly observed under alkaline conditions. During extraction, the half-lives of podophyllotoxin and deoxypodophyllotoxin in methanol (100%) at room temperature were about 60 and 12 h, respectively. These two compounds showed no sign of degradation, either for standard or in an extracted sample, when they were dried and stored at −80 °C, even for a period of 6 months. Resolubilization of the extract in 70% (v/v) aqueous ethanol offered a better stability of podophyllotoxin and deoxypodophyllotoxin.

The effect of incubation temperature was also evaluated, and no significant differences were observed whatever the temperature used (Table 2).

Sequential extraction performed three times did not increase the yield of deoxypodophyllotoxin, but a second extraction allowed for the recovery of around 10% more podophyllotoxin, bringing the total recovered concentration of podophyllotoxin to 24.4 mg/g DW.

Additional treatments such as alkaline hydrolysis and enzymatic digestion using cellulase or β-glucosidase (2 units/mL in 0.05 M phosphate buffer, pH4.8), which have been shown to allow better extraction of another lignan, secoisolariciresinol,[22] did not increase the extraction yields of podophyllotoxin and deoxypodophyllotoxin (data not shown). This suggests that podophyllotoxin and deoxypodophyllotoxin are not stored in a complex, as is the case for secoisolariciresinol in flaxseed, but as free aglycones, with a possible vacuolar localization as is frequently observed for toxic compounds.

Typical HPLC (at 280 nm) chromatograms of podophyllotoxin standard (A), deoxypodophyllotoxin standard (B), and methanol extract of leaves from *J. bermudiana* (C) as well as MS assignment of these compounds (m/z 437.2 $[M + Na]^+$) for podophyllotoxin and m/z 421.2 $[M + Na]^+$) for deoxypodophyllotoxin are shown in Figure 2.

Validation of the Method. The linear correlations between peak areas and standard concentrations were satisfying in the range of 0.125−1 mg/mL for both podophyllotoxin and deoxypodophyllotoxin. The r values for podophyllotoxin and deoxypodophyllotoxin for a five-point calibration graph were >0.999, and the slope of five replicates of the calibration graph covering

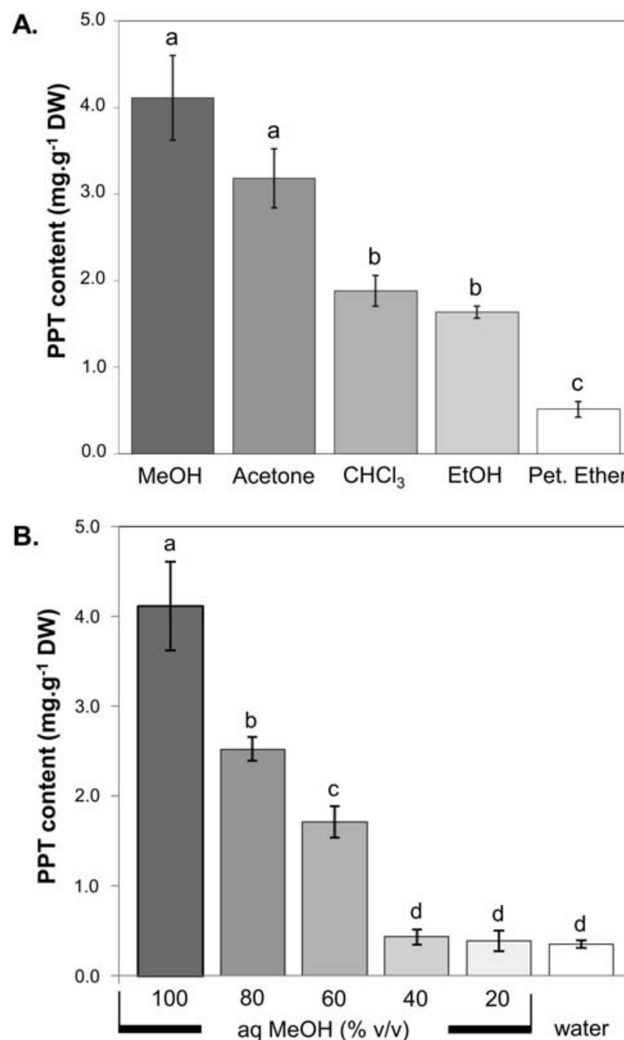

Figure 3. Podophyllotoxin (PPT) extraction from freeze-dried leaves of *J. bermudiana* as a function of (A) extraction solvent type (MeOH, methanol; $CHCl_3$, chloroform; EtOH, ethanol; Pet. Ether, petroleum ether) or (B) concentration of methanol/water ratios (% v/v) for hydroalcoholic extraction. Values are the mean ± SD of three replicates; the same letter indicates that values are not statistically different ($P > 0.05$).

the analytical range for each standard varied no more than 1% in terms of RSD over a period of 4 weeks. The LODs (S/N = 3) and LOQs (S/N = 10) were 2.9 and 9.8 μg/mL for podophyllotoxin and 0.15 and 0.50 μg/mL for deoxypodophyllotoxin, respectively.

The Supporting Information shows the results for precision, repeatability, and stability of the optimized extraction protocol. To evaluate the instrumental precision, five injections of the same extracted sample were performed. The chromatographic method was proven precise with RSDs of 2.75 and 2.11% for podophyllotoxin and deoxypodophyllotoxin, respectively. Repeatability was evaluated by applying the whole extraction procedure five times to the same starting material. The obtained RSD values were low (4.19 and 6.17% for podophyllotoxin and deoxypodophyllotoxin, respectively). The stability was determined by five injections with the same sample over 72 h (0, 6, 12, 24, 48, and 72 h after extraction). A good stability of the extracted sample was observed with low RSD values of 1.77 and 4.15% for

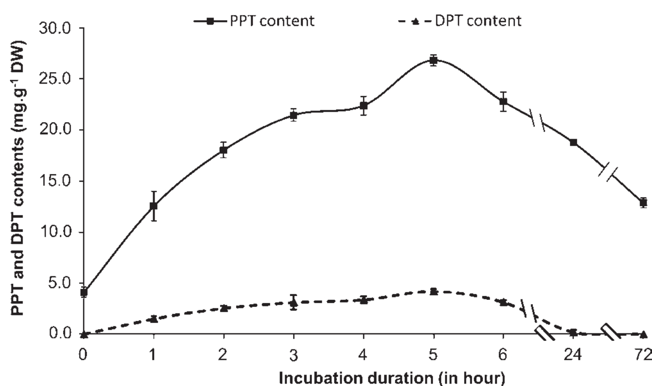

Figure 4. Podophyllotoxin (PPT) and deoxypodophyllotoxin (DPT) extractions from *J. bermudiana* freeze-dried leaves as a function of extraction duration with methanol (100%). Values are the mean ± SD of three replicates.

podophyllotoxin and deoxypodophyllotoxin, respectively. The RSDs of retention times during the validation procedure were satisfactory (0.15% for podophyllotoxin and 0.07% for deoxypodophyllotoxin). Finally, the accuracy of the method was evaluated by the standard addition procedure with two addition levels of 50 and 150 μg of podophyllotoxin as well as 5 and 15 μg of deoxypodophyllotoxin. The standard mixture was added to the sample, and the whole extraction procedure was carried out. The results demonstrated a good recovery of the compounds under study (ranging from 98.61 to 98.80% for podophyllotoxin and from 96.12 to 103.08% for deoxypodophyllotoxin).

This optimized extraction protocol for podophyllotoxin and deoxypodophyllotoxin from leaves of *J. bermudiana* was proven to be satisfactory in terms of precision, repeatability, stability, and accuracy and was further used to investigate podophyllotoxin and deoxypodophyllotoxin contents in dried aerial parts of 12 *Juniperus* species.

Podophyllotoxin and Deoxypodophyllotoxin Contents in 12 Other *Juniperus* Species. So far, podophyllotoxin and deoxypodophyllotoxin have been found in some *Juniperus* species, although in most cases at low levels, probably because extraction protocols deserved optimization. Our optimized extraction protocol for podophyllotoxin and deoxypodophyllotoxin was therefore assayed on leaves of *J. bermudiana*, *J. communis*, *J. conferta*, *J. deppeana*, *J. flaccida*, *J.* × *media*, *J. phoenicea*, *J. procumbens*, *J. sabina*, *J. scopulorum*, *J. squamata*, and *J. virginiana*. For all species tested, the assignment of podophyllotoxin and deoxypodophyllotoxin was facilitated by electrospray tandem mass spectrometry (LC-ESI-MS).

Podophyllotoxin was previously detected at low levels (0.005−0.06 mg/g DW; Table 1) in *J. chinensis*,[23] *J. communis*, *J. squamata*, and *J. recurva*[24] and at higher levels (1−4.8 mg/g DW; Table 1) in *J.* × *media*,[24] *J. sabina*, *J. scopulorum*,[16] and *J. virginiana*.[16,25−27]

In our hands, podophyllotoxin contents varied from 1.8 to 22.6 mg/g DW (Table 3) in *J. phoenicea* and *J. bermudiana*, respectively. Podophyllotoxin was quantified for the first time in *J. bermudiana* (22.6 mg/g DW; Table 3) and in *J. phoenicea* (1.8 mg/g DW; Table 3). The 22.6 mg/g DW (even 24.4 mg/g DW if re-extracted) of podophyllotoxin measured in *J. bermudiana* constitutes by far the highest podophyllotoxin content measured in *Juniperus* species. Only trace amounts of podophyllotoxin were detected in *J. squamata*, whereas no podophyllotoxin was detected in *J. deppeana*, *J. flaccida*, *J. martinezii*, and *J. scopulorum* (Table 3).

Table 2. Effect of Incubation Temperature on Extraction Capacity of Podophyllotoxin and Deoxypodophyllotoxin from *J. bermudiana* Leaves with Methanol (100%)[a]

temperature (°C)	podophyllotoxin content (mg/g DW)	deoxyodophyllotoxin content (mg/g DW)
4	21.7 ± 1.0 abc	4.2 ± 0.3ab
25	22.7 ± 0.5 abc	4.4 ± 0.3 ab
37	21.5 ± 0.4 abc	4.1 ± 0.1 ab
50	19.5 ± 0.6 ab	3.3 ± 0.3 c
60	21.4 ± 1.5 abc	3.7 ± 0.1 ac
75	20.8 ± 0.2 ac	3.9 ± 0.1 ac

[a] Values are the mean ± SD of three replicates; the same letter indicates that values are not statistically different ($P > 0.05$).

Table 3. Podophyllotoxin and Deoxypodophyllotoxin Contents in Leaves of 12 *Juniperus* Species Measured in the Present Study[a]

species	podophyllotoxin content (mg/g DW)	deoxypodophyllotoxin content (mg/g DW)
J. bermudiana	22.6 ± 0.5	4.7 ± 0.3
J. communis	0.2 ± 0.1	1.1 ± 0.1
J. conferta	nd	nd
J. deppeana	nd	nd
J. flaccida	nd	nd
J. martinezii	nd	nd
J. × *media*	4.3 ± 0.2	3.91 ± 0.1
J. phoenicea	1.8 ± 0.1	0.4 ± 0.1
J. procumbens	nd	5.8 ± 0.5
J. sabina	2.3 ± 0.1	1.8 ± 0.2
J. scopulorum	nd	nd
J. squamata	tr	4.8 ± 0.3
J. virginiana	17.8 ± 1.3	3.6 ± 0.9

[a] Values are the mean ± SD of three replicates. nd, not detected; tr, traces.

Deoxypodophyllotoxin was previously found at very low levels (0.005−0.02 mg/g DW; Table 1) in *J. blaaws*[23] and *J. procumbens*[24] and at higher concentrations ranging from 1.3 to 3.3 mg/g DW (Table 1) in *J. sabina*,[21] *J. recurva*, *J. squamata*, *J. communis*, and *J.* × *media*.[24] Deoxypodophyllotoxin was also detected but not quantified (Table 1) in *J. bermudiana*,[28] *J. phoenicea*,[29] and *J. taxifolia*.[12]

In the present study, deoxypodophyllotoxin concentrations varied considerably among the species analyzed. When detected and quantified, the optimized extraction protocol allowed the extraction of deoxypodophyllotoxin ranging from 0.4 to of 5.8 mg/g DW from leaves of *J. phoenicea* and *J. procumbens*, respectively (Table 3). High amounts of deoxypodophyllotoxin were also detected in *J. squamata* (4.8 mg/g DW; Table 3), *J. bermudiana* (4.4 mg/g DW; Table 3), and *J. sabina* (1.8 mg/g DW; Table 3). Deoxypodophyllotoxin was also detected and quantified for the first time in *J. virginiana* leaves (3.6 mg/g DW of deoxypodophyllotoxin; Table 3). The presence of

Table 4. Interindividual and Interyear Variations of Podophyllotoxin and Deoxypodophyllotoxin Concentrations in the Leaves of *J. virginiana* Collected in the Arboretum of Villa Thuret (INRA Juan-les-Pins, France)[a]

	interindividual variations (mg/g DW)			interyear variations (mg/g DW)	
	1	2	3	1, 2009	1, 2010
podophyllotoxin	16.9 ± 1.4	17.5 ± 2.5	17.8 ± 1.3	15.8 ± 0.7	16.9 ± 1.4
deoxypodophyllotoxin	4.1 ± 0.3	4.6 ± 0.5	3.6 ± 0.9	4.5 ± 0.2	4.1 ± 0.3

[a] Values are the mean ± SD of three replicates.

deoxypodophyllotoxin was not shown in *J. deppeana*, *J. flaccida*, *J. martinezii*, and *J. scopulorum* (Table 3).

Except for the report of Kusari et al.,[24] no *Juniperus* was reported to contain both podophyllotoxin and deoxypodophyllotoxin (Table 1); this could be due to the extraction protocols that did not allow the recovery of both compounds.

In our hands, *J. virginiana* yielded high amounts of both podophyllotoxin (17.8 mg/g DW; Table 3) and deoxypodophyllotoxin (3.6 mg/g DW; Table 3), and *J. bermudiana* yielded even more podophyllotoxin and deoxypodophyllotoxin (22.6 and 4.4 mg/g DW, respectively; Table 3). *J. sabina* and *J. phoenicea* contained both podophyllotoxin and deoxypodophyllotoxin at lower but non-negligible levels (Table 3). *J. squamata* accumulates more deoxypodophyllotoxin (4.8 mg/g DW; Table 3) than podophyllotoxin (traces; Table 3), which could be due to the lack of efficient enzymatic conversion of deoxypodophyllotoxin to podophyllotoxin in this *Juniperus* species.

Interindividual and seasonal variations in podophyllotoxin and deoxypodophyllotoxin contents were also investigated using *J. virginiana* growing in the Arboretum of Villa Thuret (INRA Juan-les-Pins, France): three individuals and two different years were analyzed. The results are presented in Table 4 and indicate no significant variations in podophyllotoxin or deoxypodophyllotoxin contents. These results are in accordance with two previous studies carried out on interindividual and seasonal variations in podophyllotoxin content in *J. virginiana* growing in the (U.S.) state of Mississippi.[26,27] These authors emphasized the absence of significant interaction between plant type and sampling date and showed the collection of plant material for commercial extraction of podophyllotoxin can be done independently of the collection site.

We have described the development and validation of an efficient extraction procedure followed by HPLC-based separation and quantification protocol that were developed for podophyllotoxin and deoxypodophyllotoxin content analysis of *Juniperus* species leaves. This method clearly showed its suitability for podophyllotoxin and deoxypodophyllotoxin analysis in terms of precision, repeatability, stability, and accuracy. Using our optimized protocol podophyllotoxin levels up to 22.6 mg/g for *J. bermudiana* and 17.8 mg/g for *J. virginiana* were recovered, corresponding to approximately 4-fold increases over the best published yields. Moreover, interesting levels of deoxypodophyllotoxin, a potentially useful related compound, were also found in species such as *J. procumbens*, *J. squamata*, *J. bermudiana*, *J. × media*, and *J. virginiana*. Given these high accumulations, it could be reasonable to consider *J. virginiana* and *J. bermudiana* leaves as potential alternative sources of the podophyllotoxin molecule currently extracted from the rhizome of the *Podophyllum* plant, threatened by its current overcollection. These two *Juniperus* species present the advantage of being tall trees (and not spreading shrubs; *J. virginiana* can grow up to 18 m in height and 1 m in diameter). Moreover, in these *Juniperus* species podophyllotoxin is extractible from the easily harvested aerial part; pruning can yield raw material for extraction in a sustainable way. Nevertheless, *J. bermudiana*, despite its high podophyllotoxin and deoxypodophyllotoxin contents, is not currently a promising source because it is threatened by extinction due to high lethality caused by two pathogenic insects. However, crossing it with *J. virginiana* is feasible,[30] and in vitro cloning of remaining resistant trees could be possible as in vitro culture has been successful in other *Juniperus* species.[31] This drawback is not observed with *J. virginiana* as it is even considered to be an invasive species and grown for its wood. For this reason large amounts of foliage can be recovered as a byproduct of the timber industry, making *J. virginiana* a promising alternative source of podophyllotoxin and related compounds for the pharmaceutical industry.

ASSOCIATED CONTENT

Supporting Information. Additional tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

LC-ESI-MS, liquid chromatography electrospray ionization mass spectrometry; MS, mass spectrometry; m/z, mass to charge ratio; RP-HPLC, reversed-phase high-performance liquid chromatography; RT, retention time.

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