

Article

Green Ultrasound-Assisted Extraction of Podophyllotoxin from *Juniperus scopulorum* Needles

Catherine Verret, Anja Rakotondramavo and Sullivan Renouard *

CNRS, Bordeaux INP, CBMN, UMR 5248, University of Bordeaux, Allée G. St Hilaire, F-33600 Pessac, France; catherine.verret@u-bordeaux.fr (C.V.); anja.rakotondramavo@u-bordeaux.fr (A.R.)

* Correspondence: sullivan.renouard@u-bordeaux.fr

Abstract: Background—Podophyllotoxin, a polyphenolic compound with major applications in cancer treatment, is in short supply, as its source is now an endangered plant species. It is therefore essential to find another available plant that produces high levels of podophyllotoxin. Some *Juniperus* species are known to contain podophyllotoxin, more exactly within their needles, but are still unused in this regard. Objective—The aim is to develop an efficient green ultrasound-assisted extraction protocol for podophyllotoxin from commercially available *Juniperus scopulorum* varieties. Methodology—To highlight optimal extraction conditions, a single-factor experiment was performed to identify the parameters (extraction duration, frequency, temperature extraction, preliminary grinding, and plant material concentration) influencing extraction. A multifactorial approach using the Box–Behnken design was then applied to determine the exact optimal extraction conditions. Results—The conditions for extracting the maximum amount of podophyllotoxin consist of 51.5 min of extraction time, in ethanol at 69.3%, and at 58.8 °C. This is the highest podophyllotoxin extraction yield ever obtained from *Juniperus scopulorum* needles. Compared with the reference protocol for the extracting of podophyllotoxin from *Juniperus*, ultrasonic extraction is an eco-friendly protocol, safe for humans, and the podophyllotoxin extracted by this method has much greater stability, allowing its purification. The analyses also indicate a variation in the podophyllotoxin content of *Juniperus scopulorum* needles, (from 7.02 to 10.34 mg/g DW) depending on the variety and year of purchase. Conclusion—Extraction in ethanol at 69.3% and at 58.8 °C for 51.5 min allows the extraction of up to 10.34 mg podophyllotoxin/g from freeze-dried *Juniperus scopulorum* needles.



Citation: Verret, C.;

Rakotondramavo, A.; Renouard, S. Green Ultrasound-Assisted Extraction of Podophyllotoxin from *Juniperus scopulorum* Needles. *Appl. Sci.* **2023**, *13*, 12194. <https://doi.org/10.3390/app132212194>

Academic Editor: Dino Musmarra

Received: 6 September 2023

Revised: 3 November 2023

Accepted: 6 November 2023

Published: 10 November 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Keywords: green extraction; *Juniperus scopulorum* needles; podophyllotoxin; ultrasonic extraction

1. Introduction

Podophyllotoxin is a lignan, a polyphenol, a natural plant metabolite (Figure 1) [1] isolated in the 19th century [2–5] and presenting remarkable properties: mitotoxic, neurotoxic, insecticidal, antimicrobial, anti-inflammatory, antispasmodic, hypolipidemic, immunosuppressive, antioxidative, analgesic, and cathartic activities [6].

Furthermore, podophyllotoxin is the unique molecule used as a precursor for the hemisynthesis of some anticancer drugs: etoposide, teniposide, or etopophos with topoisomerase II inhibition ability [7–16]. These anticancer drugs are involved in the treatment of certain cancers, including lung, breast, testicular, gastric, and blood [17–19].

The literature also attributes antiviral activity to podophyllotoxin against *Herpes simplex* type 1 [20], to some derivatives against HIV [21], and etoposide can fight cytokinin storms in patients infected with the COVID-19 virus [22].

Despite its clinical importance, the supply of podophyllotoxin is currently problematic. Podophyllotoxin is classically extracted from the rhizomes of *Podophyllum peltatum* or *Sinopodophyllum hexandrum* Royle [1,23], yielding up to 4% DW podophyllotoxin [24]. However, with slow regeneration and without organized cultivation, the *Sinopodophyllum hexandrum* Royle overexploitation (due to its podophyllotoxin) has led the plant to

being classified as “critically endangered”, in Appendix II of The Convention on International Trading of Endangered Species (CITES). Although complete chemical synthesis of podophyllotoxin is possible, the complex structure makes it economically unviable [15].

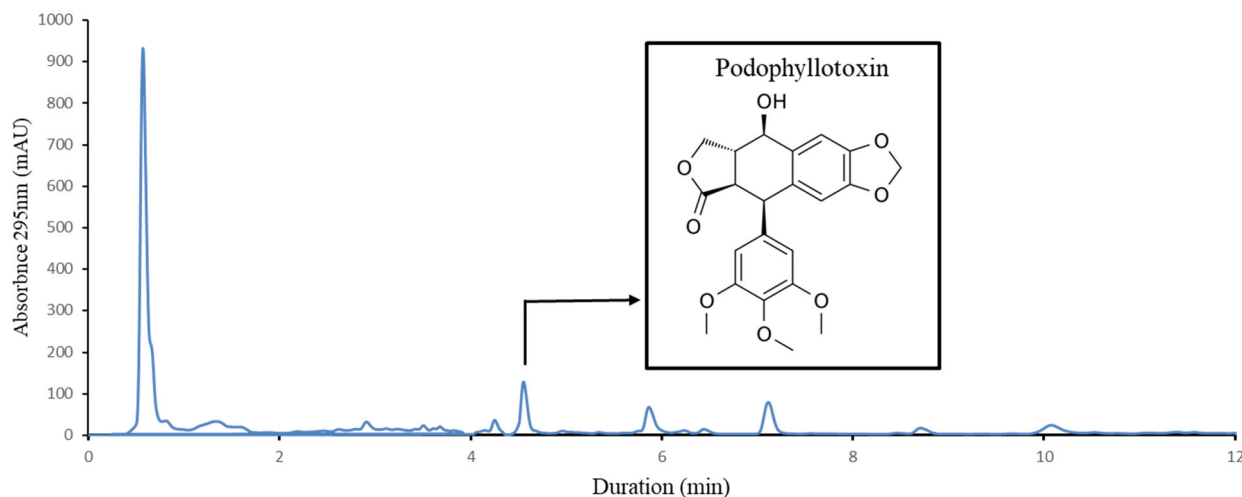


Figure 1. HPLC chromatogram of *J. scopulorum* extract and chemical structure of podophyllotoxin.

Thus, since 2018, there has often been a shortage of etoposide injections, a problem most often solved by switching to etopophos injections [25]. However, without podophyllotoxin available, there is no way to produce etoposide or etopophos, and consequently, cancer patients will no longer be able to receive the treatment they vitally need. Therefore, to continue to ensure the future podophyllotoxin needs, one promising idea is to find other biological materials as plant sources containing a significant amount of podophyllotoxin.

The *Juniperus* genus appears to be interesting as an alternative natural source of podophyllotoxin. The occurrence of cytotoxic lignan (especially podophyllotoxin) has been described in the needles of *Juniperus* species since 1953 [26], then confirmed in *Juniperus virginiana* [27–33], *Juniperus × media* [31–34], *Juniperus rigida* [35–37], *Juniperus horizontalis* [33,38], *Juniperus sabina* [31,34,39], and other species including *Juniperus scopulorum* [31–33]. The most interesting species is *Juniperus bermudiana*, with 22.6 mg podophyllotoxin/g DW [31]. However, this species is endangered and protected by the International Union for Conservation of Nature (“<https://www.iucn.org>” (accessed on 2 November 2023)). Although *Juniperus* needles don’t contain as much podophyllotoxin as *Sinopodophyllum hexandrum* rhizomes, some *Juniperus* yet contain around 2% DW podophyllotoxin [31], which is the most credible amount found in an alternative natural source. In addition, some *Juniperus* species are suitable for cultivation, providing a greater amount of plant material than *Podophyllum* [33]. *Juniperus* is therefore a potentially creditable source of podophyllotoxin. Among all the *Juniperus* species, *Juniperus scopulorum* has not been extensively studied even though it contains podophyllotoxin [31–33]. Moreover, it has several cultivated varieties, including *Juniperus scopulorum* Blue Arrow and *Juniperus scopulorum* Skyrocket. Thus, this work proposes to explore the credibility of cultivated varieties of these two *Juniperus scopulorum* as a source of podophyllotoxin.

However, studying the podophyllotoxin content of these plants requires the development and optimization of an efficient extraction protocol. Ultrasonic-assisted extraction appears to be the smartest choice, as this non-conventional technology requires less extraction solvent, a shorter extraction duration, and less thermal degradation while offering higher extraction yield than conventional approaches [40,41]. Additionally, this extraction method is suitable with the use of a green solvent (e.g., ethanol, which also solubilizes phenolic compounds), enabling green extraction. Green extraction is often the preferred extraction approach since it is safer for the environment and human health.

In the ultrasonic extraction approach, thermal, mechanical, and cavitation effects are used to extract the bioactive components from the cells. Indeed, above 20 kHz, ultrasonic

radiation extraction of phenolic compounds from solid matrices using liquid solvents creates cavitation bubbles close to the plant cells, which collapse, rupturing the cell wall and cell membrane, and releasing the cell contents. Sonication time, temperature, solvent selection, wave frequency, and ultrasonic wave distribution affect the extraction. The probe and bath are the two main systems currently used, but the ultrasonic bath offers better reproducibility and repeatability and limits the problem of sample contamination and foam production [42–47]. Therefore, we herein preferred to use an ultrasonic bath.

The literature supports the value of ultrasound for podophyllotoxin extraction: ultrasonic pretreatment can help to extract podophyllotoxin from *Podophyllum peltatum* rhizomes without podophyllotoxin degradation [48]. Next, Corbin et al. (2015) [49] developed the most efficient extraction protocol for secoisolariciresinol (a precursor of podophyllotoxin) from flax seeds using ultrasound. However, to date, there is no protocol for extracting podophyllotoxin from *Juniperus* needles using ultrasound. Therefore, it is interesting to develop this extraction approach.

Considering all this data, green ultrasound-assisted extraction of podophyllotoxin from cultivated varieties of *Juniperus scopulorum* will be developed in this work. First, single-factor experiments will identify the factors affecting the extraction. Then, a multifactorial approach will be developed to determine optimal extraction conditions. Finally, the optimized protocol will be applied to different *Juniperus scopulorum* varieties to compare its efficiency with the reference protocol and measure the stability of the extracted podophyllotoxin.

This study aims to determine the interest in *J. scopulorum* as a new source of podophyllotoxin (for the pharmaceutical industry) and the credibility of ultrasonic-assisted extraction to achieve it.

2. Materials and Methods

2.1. Plant Material

Juniperus scopulorum Blue Arrow and *Juniperus scopulorum* Skyrocket plants were obtained from Promesse de fleurs (Houplines, France). Some plants were purchased in 2019 and others in 2021. The plants were kept in pots for one month in a phytotron room at 25 °C under a 12 h photoperiod. Plant irrigation was conducted via the water reservoir of the pots. After one month, needles were collected, stored for 48 h at −80 °C, and then lyophilized for 72 h under secondary vacuum (temperature below 50 °C and pressure below 0.12 mbar) using a CHRIST ALPHA 2-8 LD-plus apparatus coupled with a vacuum pump. The lyophilized needles were used as the starting material for extraction.

2.2. Chemicals

Podophyllotoxin standard of over 98% purity was purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Analytical grade extraction solvents of over 99.9% purity were purchased from Grosseron (Saint-Quentin-Fallavier, France).

2.3. Grinding Approach

Some (lyophilized) samples underwent preliminary mechanical grinding on an IKA A11 mill equipped with a stainless steel beater. The samples were ground twice for 30 s at 28,000 rpm. The powder was then passed over a 60 mesh sieve to obtain particles of 250 µm in size.

2.4. Ultrasound-Assisted Extraction (USAE) Optimization

USAE was conducted using an electronic bath, Elmasonic P30H, with an inner dimension of 240 × 100 × 137 mm, electrical power of 320 W, maximal heating power of 320 W, variable frequencies (0, 37, and 80 kHz), and equipped with a digital timer and a temperature controller. The samples (5/10/20 mg) were placed in a 1.5 mL polypropylene tube and suspended in a corresponding volume of aqueous ethanol. During the ultrasonic treatment, the tubes were maintained immersed in the bath by a float. To conduct the study, various

material concentrations were tested (10, 20, and 50 g/L), and several aqueous ethanol concentrations were used (40, 50, 60, 70, 80, 90, and 100% (v/v)). Also, different extraction durations (10, 20, 30, 40, 60, and 80 min), several extraction temperatures (20, 40, 60, and 80 °C), and three ultrasound frequencies (0, 37, and 80 kHz) were tested. Additionally, the impact of a preliminary grinding (made with a mechanical grinder) was evaluated. The extracts were filtered (0.45 µm) before HPLC analysis.

2.5. Reference Solid/Liquid Extraction

The Renouard et al. (2011) [31] protocol, which offers the highest podophyllotoxin extraction yield from *Juniperus* needles, was used as a reference. In brief, the *Juniperus* needles are ground in absolute methanol using an Ultraturax (Ultraturax T25 basic) for 3 min at 19,000 rpm with a mass/volume ratio of 10 mg/mL. The extract is then left to macerate for 5 h under agitation at 25 °C. The solid residue is then centrifuged (15 min—3000 rpm) to retain only the supernatant containing the podophyllotoxin.

2.6. High-Performance Liquid Chromatography (HPLC) Analysis

The podophyllotoxin quantification was carried out on an Agilent liquid chromatographic system including an Agilent 1260 Infinity G5611A Quaternary Pump (with an include degasser), an Agilent 1260 Infinity II G7129A vial sampler, and an Agilent 1260 Infinity II G7117C diode array detector (DAD). The entire HPLC chain was controlled by HPLC 1260 Infinity software. The separation was performed at 35 °C on a KINETEX (Phenomenex) F5 column (100 × 3.0 mm i.d; 2.6 µm), with 5 µL as the injection volume. The mobile phase was composed of HPLC-grade solvents: water (solvent A) and methanol (solvent B). With a flow rate of 0.7 mL/min, a nonlinear gradient was applied for the mobile phase variation as follows: from 0 to 2 min of A–B: 70:30 (v/v) to 38:62 (v/v), from 2 to 9 min of A–B: 38:62 (v/v) to 33:67 (v/v), and from 9 to 12 min of A–B: 33:67 (v/v) to 5:95 (v/v). Then, the column was rinsed with absolute methanol for 10 min. Detection was performed at 295 nm. Podophyllotoxin was identified due to its retention time and UV spectrum in comparison to the reference standard. Quantification was performed using a calibration curve of podophyllotoxin ranging from 125 µg/mL to 1 mg/mL, with a correlation coefficient higher than 0.999, LOD = 35.73 µg/mL, and LOQ = 108.27 µg/mL.

2.7. Experimental Design

Response surface plots and factorial experiment design were used to determine the optimal podophyllotoxin extraction conditions with XLSTAT 2022.2.1 software (Addinsoft, Paris, France). The preliminary experiments allowed the assignment of the three independent variables affecting extraction yield and their range values. The variables were coded at three levels: −1, 0, and 1, and the selected values were X_1 —ethanol concentration (60, 75, and 90% (v/v)), X_2 —incubation duration (30, 55, and 80 min), and X_3 —temperature (40, 60, and 80 °C) (Table 1). Eighteen observations of different combinations were prepared, as presented in Table 2. All the observations were carried out in five independent replicates. The equation calculation was provided by the XLSTAT2022 software (Addinsoft, Paris, France).

Table 1. Identities, code units, coded levels, and actual experimental values of the three independent variables.

Independent Variable	Code Unit	Coded Variable Level		
		−1	0	1
Ethanol concentration (% v/v)	X_1	60	75	90
Incubation duration (min)	X_2	30	55	80
Temperature (°C)	X_3	40	60	80

Table 2. Results of experimental design.

Run ID	Run Order	X ₁	X ₂	X ₃	PT (mg/g DW)
Obs1	11	−1	−1	0	5.9913053
Obs2	6	1	−1	0	6.51152504
Obs3	9	−1	1	0	6.69284701
Obs4	15	1	1	0	5.72977268
Obs5	14	−1	0	−1	6.20516809
Obs6	4	1	0	−1	6.48178819
Obs7	12	−1	0	1	5.83502077
Obs8	10	1	0	1	6.09834046
Obs9	17	0	−1	−1	6.40098953
Obs10	8	0	1	−1	5.80521013
Obs11	5	0	−1	1	5.84521745
Obs12	18	0	1	1	6.25163654
Obs13	3	0	0	0	7.07106035
Obs14	1	0	0	0	6.88392753
Obs15	16	0	0	0	7.13525193
Obs16	2	0	0	0	6.77948881
Obs17	13	0	0	0	7.01239831
Obs18	7	0	0	0	6.79463394

2.8. Statistical Analysis

Each experiment was conducted at least 3 times. A comparative statistical analysis of groups was performed using the Student's *t*-test. The statistical tests were significant at $p < 0.05$. The graphical and statistical treatments were performed using Microsoft Excel 2010 software, except for the experimental design, which was performed using XLSTAT2022 software (Addinsoft, Paris, France).

3. Results

3.1. Preliminary Single Factor Experiments

Single-factor experiments were chosen to evaluate the relative impact of different conditions on the extraction yield of podophyllotoxin from *Juniperus scopulorum* needles. Previously, we developed an HPLC separation gradient enabling precise identification and quantification of podophyllotoxin (Figure 1). The impact of different parameters identified as important in the literature was tested: extraction duration, ultrasound frequency, ethanol concentration, solvent/material ratio, prior grinding, and extraction temperature. These preliminary single-factor experiments determined the main limiting factors. In the second stage, in order to measure the direct impact and the interaction effect between these parameters, they will be applied in the design of the experiments.

On the other hand, as the objective is to develop a green extraction protocol, an extraction solvent suitable for green chemistry must be chosen. Conventionally, different organic solvents can be used to extract podophyllotoxin from *Juniperus* needles: methanol, ethanol, and chloroform [31]. Ethanol appears to be the best choice because it is non-toxic, has minimal environmental impact, and can be mixed with water to improve its ability to solubilize podophyllotoxin.

As there is no protocol for podophyllotoxin USAE, we determine the parameter ranges to test based on the secoisolariciresinol USAE study [49]. For secoisolariciresinol, the optimal content is obtained with an extraction time of 60 min and a sample/solvent concentration of 5 mg/mL. Therefore, for the podophyllotoxin USAE, we tested an extraction time of up to 80 min and a sample/solvent concentration from 5 to 20 mg/mL. As the optimal solvent concentration was not exactly determined for secoisolariciresinol USAE, we tested from 40% to 100% ethanol, considering podophyllotoxin solubility. For the other parameters, we tested the largest condition allowed by the apparatus (temperature between 20 and 80 °C, frequencies equal to 0, 37, or 80 KHz).

A preliminary experiment was conducted with several extraction durations (10, 20, 30, 40, 60, and 80 min) using fixed ultrasound frequencies (37 kHz), the aqueous ethanol concentration (80%), the plant material concentration (10 g DW/L), and temperature (40 °C), but without prior grinding. The results presented in Figure 2a highlight that increasing the extraction duration improves the podophyllotoxin extraction yield by up to 40 min and remains at this maximum for at least 80 min.

The sonication frequency can also influence extraction. Three ultrasound frequencies (0, 37, and 80 kHz) were tested with a fixed extraction duration (40 min), aqueous ethanol concentration (80%), plant material concentration (10 g DW/L), and temperature (40 °C), but without prior grinding. It seems (Figure 2b) that at 0 kHz, there is no significant podophyllotoxin extraction. Then, 37 kHz offers a slightly higher extraction yield compared to 80 kHz, due to the stability of the podophyllotoxin [49–52], with better repeatability. It can be noted that the apparatus allows only a choice between 0, 37, or 80 kHz.

It has also been established that temperature can have an impact on ultrasonic-assisted extraction. Thus, the effect of temperature was evaluated at 20, 40, 60, and 80 °C using a fixed extraction duration (40 min), plant material concentration (10 g DW/L), ultrasound frequency (37 kHz), aqueous ethanol concentration (80%), plant material concentration (10 g DW/L), and temperature (40 °C), but without prior grinding. The results (Figure 2c) show that increasing the temperature improves the extraction yield of the podophyllotoxin up to 60 °C; then, the maximum reached remains unchanged.

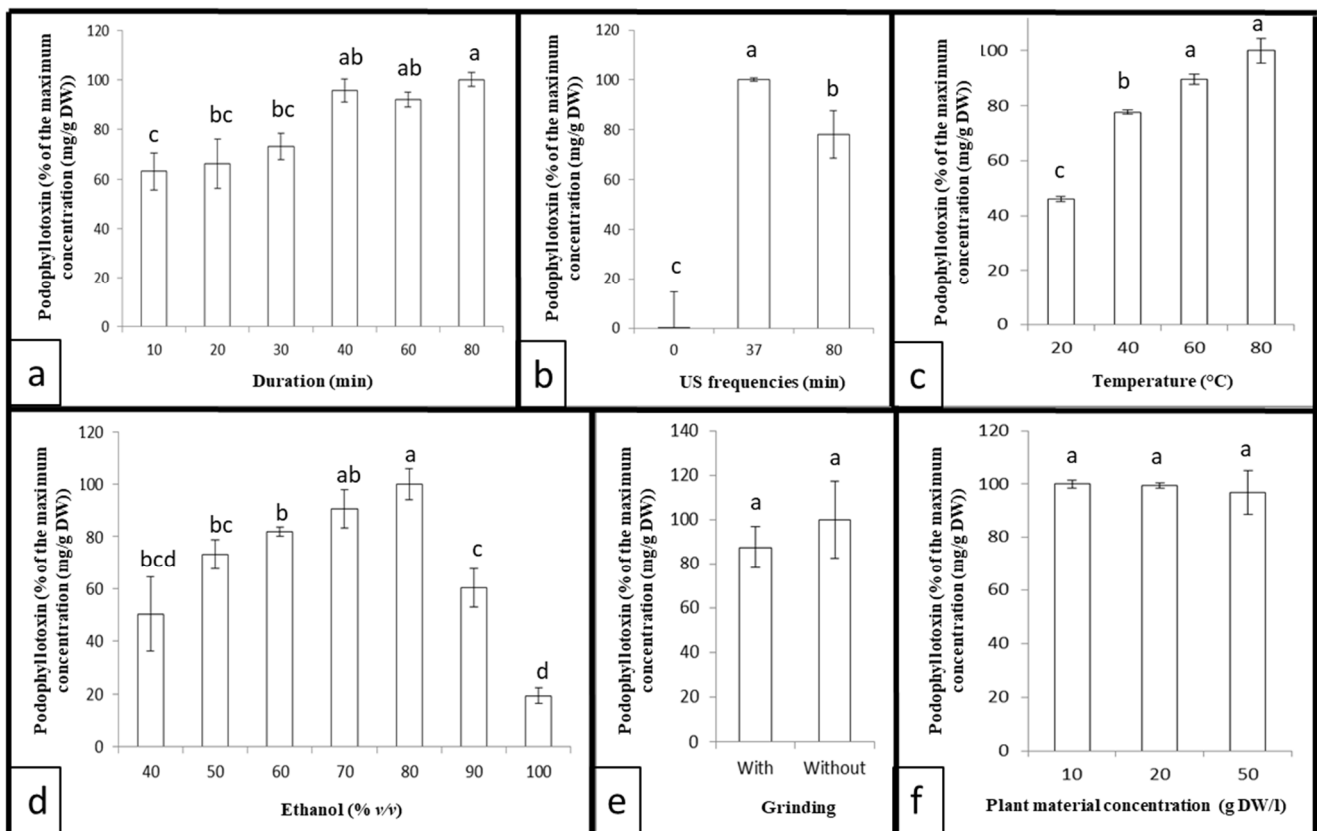


Figure 2. Podophyllotoxin content extracted from *Juniperus scopulorum* needles by a function of (a) extraction duration, (b) ultrasound frequency, (c) extraction temperature, (d) ethanol concentration, (e) grinding, and (f) plant material concentration. Values are means $\pm$ SD of 5 independent replicates. Different letters represent significant differences between the various extraction conditions ($p < 0.05$).

Different concentrations of aqueous ethanol solution were tested (40, 50, 60, 70, 80, 90, and 100% (v/v)) using a fixed extraction duration (40 min), ultrasound frequency (37 kHz), plant material concentration (10 g DW/L), and temperature (40 °C), but without prior

grinding. The extraction yield reached a maximum—increasing up to 70% and decreasing after 80% (Figure 2d).

The effect of previous grinding was analyzed using a fixed extraction duration (40 min), ultrasound frequency (37 kHz), aqueous ethanol concentration (80%), plant material concentration (10 g DW/L), and temperature (40 °C). As expected, previous grinding had no statistically significant effect on podophyllotoxin extraction yield (Figure 2e). Sonication caused cavitation bubbles that were generated close to the sample. These cavitation bubbles collapse, breaking down the cell walls and releasing the cell contents: phenolic compounds, etc. [46,48,53,54]. Sonication, therefore, makes grinding unnecessary.

The effect of various concentrations of plant material was also studied with 10, 20, and 50 g DW/L using a fixed extraction duration (40 min), ultrasound frequency (37 kHz), aqueous ethanol concentration (80%), plant material concentration (10 g DW/L), and temperature (40 °C), but without prior grinding. The results (Figure 2f) indicate that the concentration of plant material has no statistically significant impact on the podophyllotoxin extraction yield.

3.2. Development of a Multifactorial Approach

Knowing that interaction between the independent variables is possible, an experimental factorial design was performed and completed by statistical analysis and 3D surface response plots [55]. This method allows the ultrasound-assisted extraction of podophyllotoxin from *Juniperus scopulorum* needles to be optimized quickly and accurately.

Preliminary experiments have shown that the four variables affecting podophyllotoxin extraction are: extraction duration, ultrasonic frequency, aqueous ethanol concentration, and extraction temperature. Nevertheless, the frequency cannot be exactly adjusted in this ultrasonic bath. The frequency can only be set to 0, 37, or 80 kHz. Therefore, the 37 kHz frequency was chosen as it provides the highest podophyllotoxin extraction yield. Hence, the experimental factorial design will be realized considering only three variables: aqueous ethanol concentration (X_1 , ranging from 60 to 90% (v/v)), extraction duration (X_2 , ranging from 30 to 80 min), and extraction temperature (X_3 , ranging from 40 to 80 °C). The codes used for each independent variable are shown in Table 1. Moreover, based on our preliminary experiment, a concentration of 10 mg DW/L of plant material was chosen, without previous grinding.

Herein, a Box–Behnken design was performed. For the experiments, eighteen different conditions were randomized using independent process variables. Each condition consists of 5 independent replicates, and the podophyllotoxin content was determined by HPLC (Table 2).

The results show that the podophyllotoxin extracted from freeze-dried *Juniperus scopulorum* needles ranges from 5.81 mg/g DW (Obs10) to 7.14 mg/g DW (Obs15). Using multiple regression analysis, the following second-order polynomial equation was obtained: $Y = 6.946 + 0.012 \times X_1 - 0.034 \times X_2 - 0.108 \times X_3 - 0.318 \times X_1^2 - 0.371 \times X_1X_2 - 3.33 \cdot 10^{-3} \times X_1X_3 - 0.4 \times X_2^2 + 0.251 \times X_2X_3 - 0.473 \times X_3^2$ to represent the podophyllotoxin content (Y) as a function of the different variables (X_1 , X_2 , and X_3).

The results of the statistical analysis (Table 3) show that the quadratic coefficients X_1^2 , X_2^2 , and X_3^2 , as well as the interaction coefficients X_1X_2 and X_2X_3 , seem to significantly influence the extraction of podophyllotoxin from freeze-dried *Juniperus scopulorum* needles. Concomitantly, each linear coefficient (X_1 , X_2 and X_3) and the interaction coefficient X_1X_3 were not significant ($p > 0.05$). Thus, each variable (incubation duration, temperature, and percentage of ethanol) did not have a direct impact on the podophyllotoxin extraction. However, the interaction between the variables, percentage of ethanol incubation time, and incubation time–temperature induced a significant indirect impact on the extraction yield of podophyllotoxin from the freeze-dried *J. scopulorum*.

Table 3. Statistical analysis of regression coefficient.

Source	Value	SD	t	Pr > t
Constant	6.946	0.077	90.230	<0.0001
X ₁	0.012	0.067	0.182	0.860
X ₂	−0.034	0.067	−0.505	0.627
X ₃	−0.108	0.067	−1.618	0.144
X ₁ ²	−0.318	0.090	−3.520	0.008
X ₁ X ₂	−0.371	0.094	−3.933	0.004
X ₁ X ₃	−0.003	0.094	−0.035	0.973
X ₂ ²	−0.397	0.090	−4.398	0.002
X ₂ X ₃	0.251	0.094	2.657	0.029
X ₃ ²	−0.473	0.090	−5.243	0.001

Table 4 presents the results of the variance analysis (ANOVA) and the fit of the model obtained. Considering the model presenting the predicted podophyllotoxin extraction yield versus the experimentally obtained podophyllotoxin extraction yield, the high *F*-value (10.82) and the low *p*-value (1.3×10^{-3}) show that the model is highly significant. Then, the coefficient of determination, *R*² (0.924), and the adjusted *R*² (0.839) values confirm that the model for podophyllotoxin extraction yield from freeze-dried *Juniperus scopulorum* needles closely matches the predicted model. Also, the low value of the coefficient of variation (CV = 1.79%) demonstrates that the model fits the experimental values.

Table 4. ANOVA of the predicted model for USAE of podophyllotoxin from freeze-dried *Juniperus scopulorum* needles.

Source	Sum of Square	DDL	Mean of Square	F-Value	p-Value
Model	3.462	9	0.385	10.819	0.0013
Error	0.284	8	0.036		
Total corrected	3.747	17			
R ²	0.924				
R ² adjusted	0.839				
CV%	1.79				

According to the analysis, the 3D plots (Figure 3) clearly highlight that the extraction of podophyllotoxin from freeze-dried *Juniperus scopulorum* needles reached a maximum. Increasing the temperature and extraction duration leads to a decrease in podophyllotoxin content (Figure 3C), as suggested by the observed negative linear and quadratic coefficients.

Considering the adjusted second-order polynomial equation, it appears that the optimum conditions are: 69.3% ethanol concentration, 51.5 min incubation duration, and 58.8 °C temperature. Applying these conditions, a podophyllotoxin content of 7.02 mg/g DW was extracted from the freeze-dried *Juniperus scopulorum* needles.

3.3. Variation of Podophyllotoxin Content in Different Varieties of *Juniperus scopulorum*

In this study, the podophyllotoxin content of two varieties of *Juniperus scopulorum*, Blue Arrow and Skyrocket, was quantified by applying the optimal conditions previously established. Under the same conditions, the effect of the year of purchase of the two varieties on the podophyllotoxin content was evaluated. The results (Figure 4) demonstrate that the variety and year of purchase can significantly affect the podophyllotoxin content. *Juniperus scopulorum* Blue Arrow, bought in 2021, presented a podophyllotoxin content of about 7.02 mg/g DW, while the same variety bought in 2019 presented a podophyllotoxin content of about 10.26 mg/g DW. Moreover, *Juniperus scopulorum* Skyrocket, bought in 2019, presented about 10.34 mg of podophyllotoxin/g DW. In brief, this protocol allows up to 10.34 mg podophyllotoxin/g DW to be extracted from freeze-dried *Juniperus scopulorum* needles.

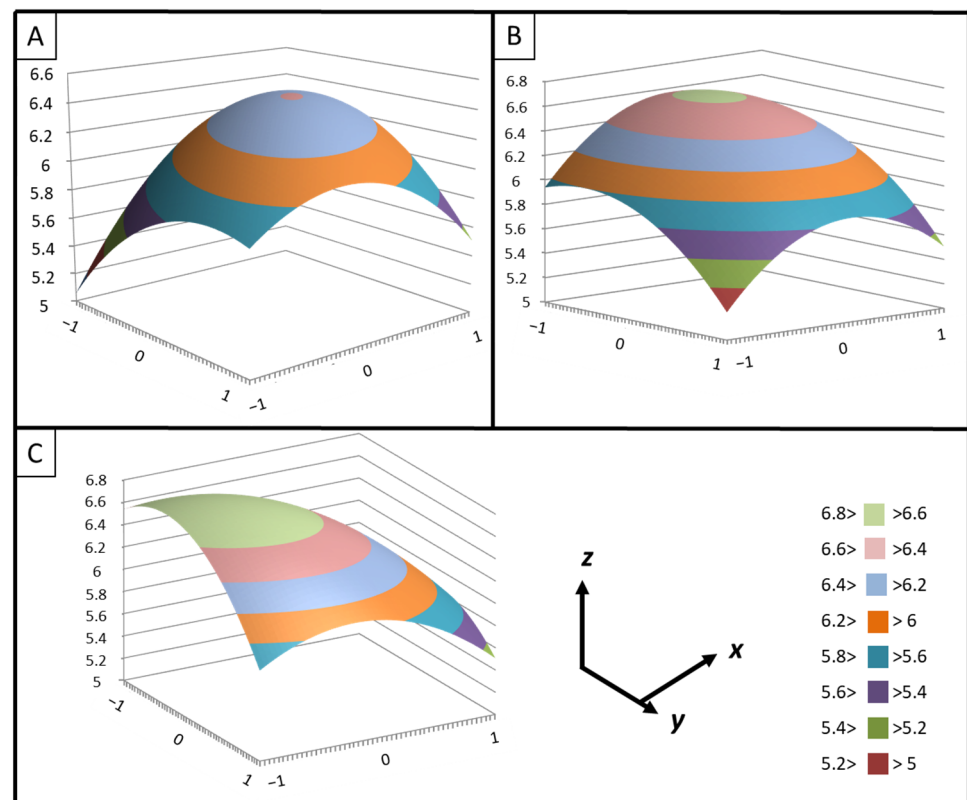


Figure 3. Predicted surface response plots of the podophyllotoxin extraction yield (y) as a function of (A): ethanol concentration (x) and extraction duration (z), (B): ethanol concentration (x) and extraction temperature (z), and (C): extraction duration (x) and extraction temperature (z).

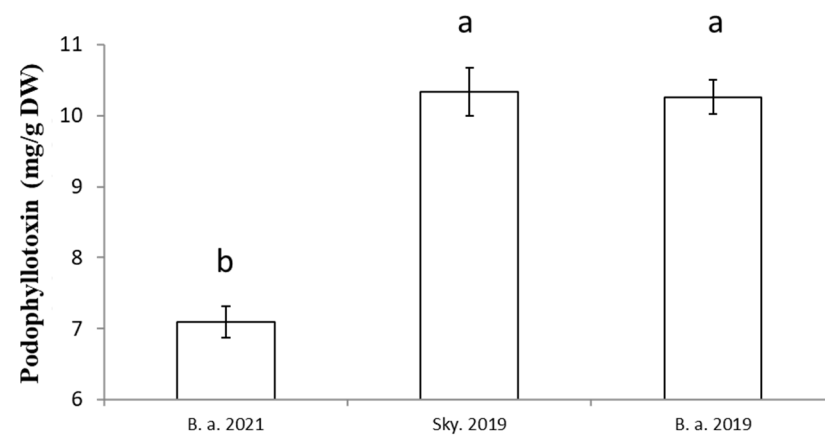


Figure 4. Comparison of podophyllotoxin content in needles (obtained using optimized extraction protocol) between several *Juniperus scopulorum* samples and/or varieties. Values are means $\pm$ SD of 5 independent replicates. Different letters represent significant differences between the various extraction conditions ($p < 0.05$).

3.4. Comparison of the Optimized Protocol with the Reference Protocol

The podophyllotoxin extraction protocol offering the best extraction yield from *Juniperus* needles known today is the protocol of Renouard et al., 2011 [31], a protocol consisting of grinding the needles using an Ultraturax in 100% methanol followed by maceration (5 h). As previously proved, podophyllotoxin content can vary between species; we tested both protocols (the optimized ultrasound-assisted protocol developed here and the Renouard et al., 2011 [31] protocol) on the same sample (*Juniperus scopulorum* Blue Arrow bought in 2021). The result (Figure 5) shows that no significant difference was detected

between these two protocols. Therefore, the ultrasound-assisted extraction protocol developed in this study is equal to the Renouard et al. (2011) [31] protocol and also offers the best extraction yield of podophyllotoxin from *Juniperus scopulorum* needles.

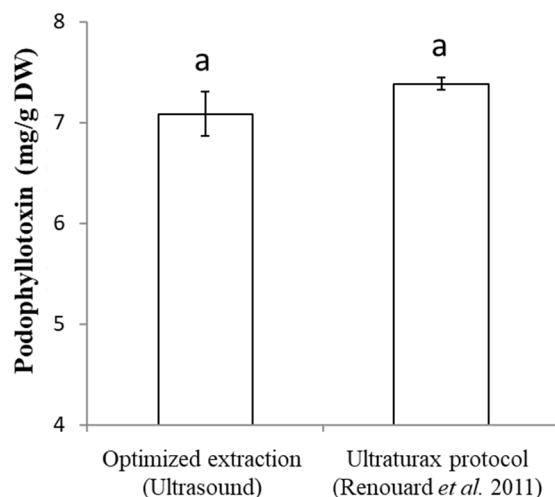


Figure 5. Comparison of podophyllotoxin content in *Juniperus scopulorum* needles obtained using the optimized extraction protocol vs. Renouard et al., 2011 protocol [31]. Values are means $\pm$ SD of 5 independent replicates. Different letters represent significant differences between the various extraction conditions ($p < 0.05$).

3.5. The *Juniperus* Extract Stability

To assess the interest in developing an extraction protocol for a specific/interesting molecule, it is crucial to consider the stability of the extracted molecule. For this purpose, it is necessary to evaluate the duration of the presence of podophyllotoxin in the extract without decreasing its content. The experiments show (Figure 6) that podophyllotoxin stays stable in the extract at 25 °C for up to 6 days. A statistically significant decrease was measured after 10 days. This is an interesting observation compared with the protocol of Renouard et al. (2011) [31], which detected a decrease after 6 h.

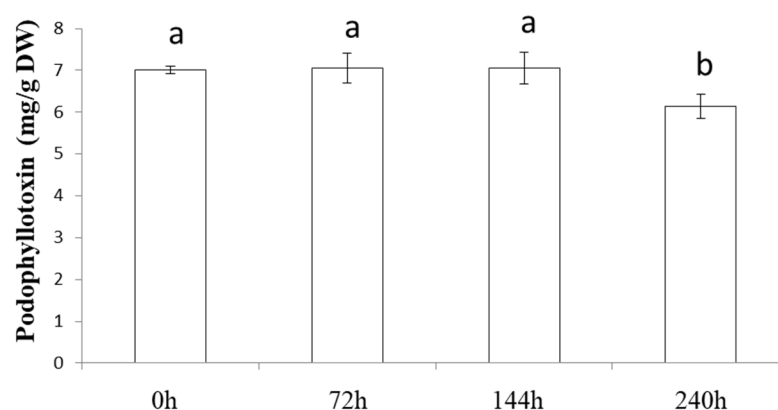


Figure 6. Stability study of podophyllotoxin content in *Juniperus scopulorum* needles obtained using the optimized extraction protocol. Values are means $\pm$ SD of 3 independent replicates. Different letters represent significant differences between the various extraction conditions ($p < 0.05$).

4. Discussion

The preliminary experiment highlights the involvement of ultrasound frequency in the podophyllotoxin extraction process (Figure 2b). The literature explains that ultrasound frequency can improve the solubility of podophyllotoxin in the extraction solvent, due to the cavitation effect and the modification of the diffusion coefficient of the targeted

molecule. Up to 60 kHz, the frequency does not affect the stability of phenolic compounds after their extraction [50]. However, at 80 KHz, a significant impact is observed. Tchabo et al. (2015) [56] also measured a decrease in total phenolic compound concentration in mulberry above and below 33.82 KHz. It has already been established that the high frequency of ultrasound can lead to the degradation of phenolic compounds and, therefore, also of podophyllotoxin. [51,52]. Additionally, a similar study conducted on secoisolariciresinol, a podophyllotoxin precursor, showed better extraction yield at 30 kHz than at 45 kHz [49], illustrating the negative impact of high frequencies on lignans. Thus, using an ultrasound frequency of 37 kHz allows a better extraction yield of podophyllotoxin compared to 0 and 80 kHz.

The multifactorial approach demonstrates that the interaction between the percentage of ethanol and incubation duration is involved in the extraction of podophyllotoxin from *Juniperus scopulorum* needles. Indeed, incubation duration has a significant impact: the longer the treatment lasts, the more cavitation bubbles emerge and collapse, and the more the proportion of molecules released from cells increases [45,47]. Concomitantly, in order to release the molecules from the cell, it is essential to solubilize the molecule. De facto, the percentage of ethanol will unduly affect the extraction yield of the podophyllotoxin. Moreover, the percentage of ethanol modifies the viscosity of the solvent, whereas the cavitation threshold depends on the viscosity of the solvent [47,57]. A modification of the cavitation threshold will lead to a modification of the incubation duration, resulting in cell perforation. Then, the collapse of the cavitation bubble is more intense at low vapor pressure, which depends on the percentage of ethanol [58]. Thus, this interaction and its impact on podophyllotoxin extraction yield seem obvious.

The interaction of temperature and incubation duration has a significant favorable impact on the extraction yield of podophyllotoxin from *Juniperus scopulorum* needles. Temperature decreases solvent viscosity and surface tension, leading to attenuating the effects of sonication [58]. Then, a positive effect of temperature is associated with an increase in cavitation bubbles [59]. As the temperature can increase the number of cavitation bubbles, the extraction duration will define how long these numerous bubbles will act (perforate the cell to extract the podophyllotoxin), explaining the benefit of the interaction between temperature and incubation duration.

In this study, the optimized protocol was established to extract up to 10.34 mg podophyllotoxin/g DW from freeze-dried *Juniperus scopulorum* needles. Protocols with podophyllotoxin extraction yields of 8.5 mg/g DW [33] and 4.9 mg/g DW [32] have been reported in the literature, but this amount of podophyllotoxin has never been extracted from *Juniperus scopulorum* needles. It should be noted that this content appears to be consistent with the published content: between 0.2 mg/g DW (*Juniperus communis*) and 22.6 mg/g DW (*Juniperus bermudiana*) [31].

However, compared with the extraction protocol that yielded the highest amount of podophyllotoxin from *Juniperus* needles [31], there is no statistically significant difference. Thus, this USAE protocol is as effective as the protocol of Renouard et al., 2011 [31], which is the reference protocol. Furthermore, this USAE protocol only requires ethanol as the organic extraction solvent, which is an eco-friendly solvent with minor toxicity for humans, unlike the Renouard et al. (2011) [31] protocol, which uses methanol, a neurotoxic solvent. Also, the Renouard et al. (2011) [31] protocol requires 5 h of maceration at 25 °C after Ultraturax grinding to extract the maximum podophyllotoxin amount, while this USAE protocol does not require prior grinding and extraction lasts less than an hour. Therefore, USAE is less time-consuming, and thus more economical. Additionally, using Ultraturax, samples must be processed one by one, while the ultrasonic bath allows treating twenty samples simultaneously. Then, the Renouard et al. (2011) [31] protocol demonstrates a statistically significant podophyllotoxin loss as early as one hour after extraction, which can pose a problem for the pharmaceutical industry, because once extracted, the podophyllotoxin must immediately be purified. However, the protocol herein developed allows the extract to be stored for 6 days without loss of podophyllotoxin, thus giving more latitude/time for

purification of the active ingredient. Furthermore, the impact of the variety and/or year of purchase of the plant on podophyllotoxin content is a result that could be expected. Indeed, Peanparkdee Patrawatt and Iwamoto (2019) [60] and Zainol et al. (2020) [61] detected differences in the content of ultrasonically extracted phenolic compounds depending on geographical origin, cultivars, or plant species. In addition, regarding podophyllotoxin content specifically, damage to *Podophyllum peltatum* leaves increases the podophyllotoxin content [62]. A difference has also been shown in the needles of different *Juniperus* varieties: for instance, the *Juniperus virginiana* variety Blue Clouds contains 384.5 mg/100 g DW, whereas the variety Kosteri contains 4.3 mg/100 g DW [32]. Additionally, podophyllotoxin is a phytoalexin, i.e., a molecule produced by the plant to protect itself and/or as a response to stress. Therefore, the content of podophyllotoxin will be modified depending on the plant's basic protective ability and/or the stresses suffered by the plant [62,63]. In this study, the significant variation in podophyllotoxin levels according to the year of purchase and/or variety shows the impact of the environment and genetics on the production of molecules. To maximize the interest in *J. scopulorum* as a source of podophyllotoxin, it could be interesting to identify varieties and environmental factors that could stimulate podophyllotoxin production, along the lines of what has been conducted for one of these precursors in flax [64] or for podophyllotoxin in *Podophyllum peltatum*, considering the way the leaves are harvested and stored [62,65].

In conclusion, it is important to remember that podophyllotoxin is a well-known precursor of anti-cancer drugs, and the current plant source, *Sinopodophyllum hexandrum*, is now prohibited for the extraction of podophyllotoxin. A new alternative plant source is therefore being sought. *Juniperus* species are known to contain podophyllotoxin in their needles. Hence, we developed a protocol for the extraction of podophyllotoxin from *Juniperus scopulorum* needles. This study focused on a green chemistry approach: ultrasound-assisted extraction to avoid environmental damage, and on commercially available *Juniperus scopulorum* varieties to ensure the availability of the plant source. The protocol developed involved extraction in 69.3% ethanol for 51.5 min at 58.8 °C and allowed up to 10.34 mg/g DW podophyllotoxin to be extracted from freeze-dried *Juniperus scopulorum* needles, equaling the extraction yield obtained with the reference protocol, although a variation in the podophyllotoxin content was measured according to the variety and year of purchase of the plant. A stable podophyllotoxin content for six days was observed, which constitutes an interesting stability period to ensure the purification of the molecule after its extraction.

Thus, in the area of availability and extraction of podophyllotoxin, this work highlights a newly available and quantitatively credible source of this active molecule in *J. scopulorum* needles. In addition, it offers an efficient, rapid, environmentally friendly ultrasonic extraction protocol using a bath that can act on several samples simultaneously, enabling it to be adapted to an industrial scale.

An interesting prospect would be to identify the genetic and environmental factors stimulating the production of podophyllotoxin by *J. scopulorum* to increase the amount of podophyllotoxin produced and therefore the gains in credibility of this plant as a source of anticancer agents.

Author Contributions: Conceptualization, S.R.; Methodology, A.R.; Validation, C.V.; Investigation, C.V., A.R. and S.R.; Writing—original draft, S.R.; Writing—review & editing, A.R. and S.R.; Supervision, C.V.; Funding acquisition, C.V. and S.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the “Ligue contre le Cancer de Dordogne France”.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

Acknowledgments: The project received, for its realization, the financial support of the “Ligue contre le Cancer de Dordogne France”.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Canel, C.; Moraes, R.M.; Dayan, F.; Ferreira, D. Podophyllotoxin. *Phytochemistry* **2000**, *54*, 115–120. [[CrossRef](#)] [[PubMed](#)]
2. Cragg, G.M.; Kingston, D.G. *Anticancer Agents from Natural Products*, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2011; p. 97.
3. Podwyssotzki, V. The active constituents of podophyllotoxin. *Pharm. J. Trans.* **1881**, *12*, 217–218.
4. Podwyssotzki, V. On the active constituents of podophyllin. *Am. J. Pharm.* **1882**, *12*, 102–115.
5. Podwyssotzki, V. Pharmakologische Studien über *Podophyllum peltatum*. *Arch. Exp. Path. Pharmacol.* **1884**, *13*, 29–52. [[CrossRef](#)]
6. Yu, X.; Che, Z.P.; Xu, H. Recent Advances in the Chemistry and Biology of Podophyllotoxins. *Chem. Eur. J.* **2016**, *10*, 1002–1006. [[CrossRef](#)]
7. Stoll, A.; Renz, J.; Wartburg, A.V. The isolation of podophyllotoxin glucoside. *J. Am. Chem. Soc.* **1954**, *76*, 3103–3104. [[CrossRef](#)]
8. Stoll, A.; von Wartburg, A.; Angliker, E.; Renz, J. The isolation of 40-Demethylpodophyllotoxin glucoside from rhizomes *podophyllum emodi* wall. *J. Am. Chem. Soc.* **1954**, *76*, 5004–5005. [[CrossRef](#)]
9. Emmenggger, H.; Stahelin, H.; Rutschmann, J.; von Wartburg, A. Chemistry and Pharmacology of podophyllum glucosides and derivatives. *I. Arzneim.* **1961**, *11*, 327–333.
10. Kuhn, M.; von Wartburg, A. Mitosis-inhibiting substances. XXI. Synthesis of epipodophyllotoxin b-D-glucopyranoside. *Helv. Chim. Acta* **1968**, *51*, 1631–1641. [[CrossRef](#)]
11. Kuhn, M.; von Wartburg, A. Mitosis-inhibiting substances. Glycosidation process. II. Glycosides of 4''-demethylepipodophyllotoxin. *Helv. Chim. Acta* **1969**, *52*, 948–955. [[CrossRef](#)]
12. Keller-Juslen, C.; Kuhn, M.; VonWartburg, A.; Staehelin, H. Synthesis and antimitotic activity of glycosidic lignan derivatives related to podophyllotoxin. *J. Med. Chem.* **1971**, *14*, 936–940. [[CrossRef](#)] [[PubMed](#)]
13. Joel, S. The clinical pharmacology of etoposide. An update. *Cancer Treat. Rev.* **1996**, *22*, 179–221. [[CrossRef](#)] [[PubMed](#)]
14. Stahelin, H.F.; von Wartburg, A. The chemical and biological route from podophyllotoxin glucoside to etoposide: Ninth cain memorial award lecture. *Cancer Res.* **1991**, *51*, 5–15. [[PubMed](#)]
15. Gordaliza, M.; Garcia, P.A.; Miguel del Corral, J.M. Podophyllotoxin: Distribution, sources, applications, and new cytotoxic derivatives. *Toxicol.* **2004**, *44*, 441–459. [[CrossRef](#)]
16. Ardalani, H.; Avan, A.; Ghayour-Mobarhan, M. Podophyllotoxin: A novel potential natural anticancer agent. *Avicenna J. Phytomed.* **2017**, *7*, 285–294. [[PubMed](#)]
17. Holm, B.; Sehested, M.; Jesen, P.B. Improved targeting of brain tumors using dextrazoxane rescue of topoisomerase II combined with supra-lethal doses of etoposide and teniposide. *Clin. Cancer Res.* **1998**, *4*, 1367–1373.
18. Ekstrom, K.; Hoffman, K.; Linne, T.; Eriksson, B.; Glimelius, B. Single-dose etoposide in advanced pancreatic and biliary cancer, a phase II study. *Oncol. Rep.* **1998**, *5*, 931–934. [[CrossRef](#)]
19. Zi, C.T.; Yang, L.; Kong, Q.H.; Li, H.M.; Yang, X.Z.; Ding, Z.T.; Jiang, Z.H.; Hu, J.M.; Zhou, J. Glucoside derivatives of podophyllotoxin: Synthesis, physicochemical properties, and cytotoxicity. *Drug Des. Dev. Ther.* **2019**, *13*, 3683–3692. [[CrossRef](#)]
20. Hammonds, T.R.; Denyer, S.P.; Jackson, D.E.; Irving, W.L. Studies to show that with podophyllotoxine the early replicative stages of herpes virus type 1 depend upon functional cytoplasmic microtubules. *J. Med. Microbiol.* **1996**, *45*, 167–172. [[CrossRef](#)]
21. Chen, S.W.; Wang, Y.H.; Jin, Y.; Tian, X.; Zheng, Y.T.; Luo, D.Q.; Tu, Y.Q. Synthesis and anti-HIV-1 activities of novel podophyllotoxin derivatives. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2091–2095. [[CrossRef](#)]
22. Maulin, P.; Eduardo, D.; Daniel, S.; Parag, D.; Ashwin, C.; Michael, B.; Roberto, C.; Gerard, C.J. Etoposide as salvage therapy for cytokine storm due to coronavirus disease 2019. *Chest* **2021**, *159*, E7–E11.
23. Hartwell, J.L.; Schrecker, A.W. Components of Podophyllin. V. The Constitution of Podophyllotoxin. *J. Am. Chem. Soc.* **1951**, *73*, 2909–2916. [[CrossRef](#)]
24. Purohit, M.C.; Bahuguna, R.; Maithani, U.C.; Purohit, A.N.; Rawat, M.S.M. Variation in podophylloresin and podophyllotoxin contents in different populations of *Podophyllum hexandrum*. *Curr. Sci. India* **1999**, *77*, 1078–1080.
25. Li, H.; Cimino, S.K. Clinical impact of the etoposide injection shortage. *J. Oncol. Pharm. Pract.* **2020**, *26*, 187–192. [[CrossRef](#)] [[PubMed](#)]
26. Hartwell, J.L.; Johnson, J.M.; Fitzgerald, D.B.; Belkin, M. Podophyllotoxin from *Juniperus* species savinin. *J. Am. Chem. Soc.* **1953**, *75*, 235–236. [[CrossRef](#)]
27. Cushman, K.E.; Maqbool, M.; Gerard, P.D.; Bedir, E.; Lata, H.; Moraes, R.M. Variation of podophyllotoxin in leaves of Eastern red cedar (*Juniperus virginiana*). *Planta Med.* **2003**, *69*, 477–478.
28. Gawde, A.; Cantrell, C.L.; Zheljaskov, V.D. Dual extraction of essential oil and podophyllotoxin from *Juniperus virginiana*. *Ind. Crops Prod.* **2009**, *30*, 276–280. [[CrossRef](#)]
29. Gawde, A.; Zheljaskov, V.D.; Maddox, V.; Cantrell, C.L. Bioprospection of Eastern red cedar from nine physiographic regions in Mississippi. *Ind. Crops Prod.* **2009**, *30*, 59–64. [[CrossRef](#)]
30. Cantrell, C.L.; Zheljaskov, V.D.; Osbrink, W.L.; Castro, A.; Maddox, V.; Craker, L.E.; Astatkie, T. Podophyllotoxin and essential oil profile of *Juniperus* and related species. *Ind. Crops Prod.* **2013**, *43*, 668–676. [[CrossRef](#)]

31. Renouard, S.; Lopez, T.; Hendrawati, O.; Dupré, P.; Doussot, J.; Falguières, A.; Ferroud, C.; Hagège, D.; Lamblin, F.; Lainé, E.; et al. Podophyllotoxin and deoxypodophyllotoxin in *Juniperus bermudiana* and 12 other *Juniperus* species: Optimization of extraction, method validation, and quantification. *J. Agric. Food Chem.* **2011**, *59*, 8101–8107. [\[CrossRef\]](#)
32. Och, M.; Och, A.; Cie'sla, L.; Kubrak, T.; Pecio, L.; Stochmal, A.; Kocki, J.; Bogucka-Kocka, A. Study of cytotoxic activity, podophyllotoxin, and deoxypodophyllotoxin content in selected *Juniperus* species cultivated in Poland. *Pharm. Biol.* **2015**, *53*, 831–837. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Ivanova, D.I.; Nedialkov, P.T.; Tashev, A.N.; Olech, M.; Nowak, R.; Ilieva, Y.E.; Kokanova-Nedialkova, Z.K.; Atanasova, T.N.; Angelov, G.; Najdenski, H.M. Junipers of various origins as potential sources of the anticancer drug precursor podophyllotoxin. *Molecules* **2021**, *26*, 5179. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Kusari, S.; Zühlke, S.; Spiteller, M. Chemometric evaluation of the anti-cancer pro-drug podophyllotoxin and potential therapeutic analogues in *Juniperus* and *Podophyllum* species. *Phytochem. Anal.* **2011**, *22*, 128–143. [\[CrossRef\]](#)
35. Woo, K.W.; Choi, S.U.; Park, J.C.; Lee, K.R. A new lignan glycoside from *Juniperus rigida*. *Arch. Pharm. Res.* **2011**, *34*, 2043–2049. [\[CrossRef\]](#)
36. Han, J.W.; Shim, D.W.; Shin, W.Y.; Kim, M.K.; Shim, E.J.; Sun, X.; Koppula, S.; Kim, T.J.; Kang, T.B.; Lee, K.H. *Juniperus rigida* Sieb. extract inhibits inflammatory responses via attenuation of TRIF-dependent signaling and inflammasome activation. *J. Ethnopharmacol.* **2016**, *190*, 91–99. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Liu, Z.; Wang, D.; Li, D.; Zhang, S. Quality evaluation of *Juniperus rigida* Sieb. et Zucc. based on phenolic profiles, bioactivity, and HPLC fingerprint combined with chemometrics. *Front. Pharmacol.* **2017**, *8*, 198. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Zheljazkov, V.D.; Cantrell, C.L.; Donega, M.A.; Astatkie, T.; Heidel, B. Podophyllotoxin concentration in Junipers in the Big Horn Mountains in Wyoming. *HortScience* **2012**, *47*, 1696–1697. [\[CrossRef\]](#)
39. Xu, S.; Li, X.; Liu, S.; Tian, P.; Li, D. *Juniperus sabina* L. as a source of podophyllotoxins: Extraction optimization and anti-cholinesterase activities. *Int. J. Mol. Sci.* **2022**, *23*, 10205. [\[CrossRef\]](#)
40. Fu, X.; Wang, D.; Belwal, T.; Xie, J.; Xu, Y.; Zou, L.; Zhang, L.; Luo, Z. Natural deep eutectic solvent enhanced pulse-ultrasonication assisted extraction as a multistability protective and efficient green strategy to extract anthocyanin from blueberry pomace. *LWT* **2021**, *144*, 111220. [\[CrossRef\]](#)
41. Yusoff, I.; Taher, Z.; Rahmat, Z.; Chua, L. A review of ultrasound-assisted extraction for plant bioactive compounds: Phenolics, flavonoids, thymols, saponins and proteins. *Food Res. Int.* **2022**, *157*, 111268. [\[CrossRef\]](#)
42. Miller, D.L.; Pislaru, S.V.; Greenleaf, J.E. Sonoporation: Mechanical DNA delivery by ultrasonic cavitation. *Somat. Cell Mol. Gen.* **2002**, *27*, 115–134. [\[CrossRef\]](#)
43. Karshafian, R.; Bevan, P.D.; Williams, R.; Samac, S.; Burns, P.N. Sonoporation by ultrasound-activated microbubble contrast agents: Effect of acoustic exposure parameters on cell membrane permeability and cell viability. *Ultrasound Med. Biol.* **2009**, *35*, 847–860. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Ohta, S.; Suzuki, K.; Miyagawa, S.; Ogino, Y.; Villacorte, M.; Wada, Y.; Yamada, G. Sonoporation in developmental biology. In *Electroporation and Sonoporation in Developmental Biology*; Nakamura, H., Ed.; Springer: Tokyo, Japan, 2009; pp. 317–326.
45. Gang, D.; Wang, J.; Dudareva, N.; Nam, K.; Simon, J.; Lewinsohn, E.; Pichersky, E. An investigation of the storage and biosynthesis of phenylpropenes in sweet basil. *Plant. Physiol.* **2001**, *125*, 539–555. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Khoddami, A.; Wilkes, M.A.; Roberts, T.H. Techniques for analysis of plant phenolic compounds. *Molecules* **2013**, *18*, 2328–2375. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Shen, L.; Pang, S.; Zhong, M.; Sun, Y.; Qayum, A.; Liu, Y.; Rashid, A.; Xu, B.; Liang, Q.; Ma, H.; et al. A comprehensive review of ultrasonic assisted extraction (UAE) for bioactive components: Principles, advantages, equipment, and combined technologies. *Ultrason. Sonochem.* **2023**, *101*, 106646. [\[CrossRef\]](#)
48. Zhao, S.; Baik, O. Application of ultrasound as pretreatment for extraction of podophyllotoxin from rhizomes of *Podophyllum peltatum*. *Ultrason. Sonochem.* **2012**, *19*, 22–31. [\[CrossRef\]](#)
49. Corbin, C.; Fidel, T.; Leclerc, E.A.; Barakzoy, E.; Sagot, N.; Falguières, A.; Renouard, S.; Blondeau, J.P.; Ferroud, C.; Doussot, J.; et al. Development and validation of an efficient ultrasound assisted extraction of phenolic compounds from flax (*Linum usitatissimum* L.) seeds. *Ultrason. Sonochem.* **2015**, *26*, 176–185. [\[CrossRef\]](#)
50. Soria, A.C.; Villamiel, M. Effect of ultrasound on the technological properties and bioactivity of food: A review. *Trends Food Sci. Technol.* **2010**, *21*, 323–331. [\[CrossRef\]](#)
51. Lavilla, I.; Bendicho, C. Fundamentals of ultrasound-assisted extraction. In *Water Extraction of Bioactive Compounds*; González, H.D., González Muñoz, M.J., Eds.; Elsevier: Amsterdam, The Netherlands, 2017; pp. 291–316.
52. Medina-Torres, N.; Ayora-Talavera, T.; Espinosa-Andrews, H.; Sánchez-Contreras, A.; Pacheco, N. Ultrasound assisted extraction for the recovery of phenolic compounds from vegetable sources. *Agronomy* **2017**, *7*, 47. [\[CrossRef\]](#)
53. Toma, M.; Vinatoru, M.; Paniwnyk, L.; Mason, T. Investigation of the effects of ultrasound on vegetal tissues during solvent extraction. *Ultrason. Sonochem.* **2001**, *8*, 137–142. [\[CrossRef\]](#)
54. Vinatoru, M. An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrason. Sonochem.* **2001**, *8*, 303–313. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Tabaraki, R.; Nateghi, A. Optimization of ultrasonic-assisted extraction of natural antioxidants from rice bran using response surface methodology. *Ultrason. Sonochem.* **2011**, *18*, 1279–1286. [\[CrossRef\]](#) [\[PubMed\]](#)

56. Tchabo, W.; Ma, Y.; Engmann, F.N.; Zhang, H. Ultrasound-assisted enzymatic extraction (UAEE) of phytochemical compounds from mulberry (*Morus nigra*) must and optimization study using response surface methodology. *Ind. Crops Prod.* **2015**, *63*, 214–225. [\[CrossRef\]](#)
57. Mason, T.; Lorimer, J. General principles. In *Applied Sonochemistry: Uses of Power Ultrasound in Chemistry and Processing*; Mason, T.J., Lorimer, J.P., Eds.; Wiley: Weinheim, Germany, 2002; pp. 25–74.
58. Santos, H.; Lodeiro, C.; Capelo-Martínez, J. The power of ultrasound. In *Ultrasound in Chemistry: Analytical Applications*; Capelo-Martínez, J.L., Ed.; Wiley: Weinheim, Germany, 2009; pp. 1–16.
59. Shirsath, S.; Sonawane, S.; Gogate, P. Intensification of extraction of natural products using ultrasonic irradiations—A review of current status. *Chem. Eng. Process. Process Intensif.* **2012**, *53*, 10–23. [\[CrossRef\]](#)
60. Peanparkdee, M.; Patrawatt, J.; Iwamoto, S. Effects of extraction conditions on phenolic content, anthocyanin content and antioxidant activity of bran extracts from Thai rice cultivars. *J. Cereal Sci.* **2019**, *86*, 86–91. [\[CrossRef\]](#)
61. Zainol, M.; Mohd Subri, I.; Zamri, I.; Mohd Zin, Z.; Faisal, A.; Mamat, H. Antioxidative properties and proximate analysis of spent coffee ground (SCG) extracted using ultrasonic methanol assisted technique as a potential functional food ingredient. *Food Res.* **2020**, *4*, 636–644. [\[CrossRef\]](#)
62. Bedir, E.; Tellez, M.; Lata, H.; Khan, I.; Cushman, K.E.; Moraes, R.M. Post-harvest and scale-up extraction of American mayapple leaves for podophyllotoxin production. *Ind. Crops Prod.* **2006**, *24*, 3–7. [\[CrossRef\]](#)
63. Renouard, S.; Corbin, C.; Drouet, S.; Medvedec, B.; Doussot, J.; Colas, C.; Maunit, B.; Bhambra, A.; Gontier, E.; Jullian, N.; et al. Investigation of *Linum flavum* (L.) hairy root cultures for the production of anticancer aryltetralin lignans. *Int. J. Mol. Sci.* **2018**, *19*, 990. [\[CrossRef\]](#)
64. Garros, L.; Drouet, S.; Corbin, C.; Decourtil, C.; Fidel, T.; Lebas de Lacour, J.; Leclerc, E.A.; Renouard, S.; Tungmunthum, D.; Doussot, J.; et al. Insight into the influence of cultivar type, cultivation year, and site on the lignans and related phenolic profiles, and the health-promoting antioxidant potential of flax (*Linum usitatissimum* L.) seeds. *Molecules* **2018**, *23*, 2636. [\[CrossRef\]](#)
65. Cushman, K.E.; Moraes, R.M.; Gerard, P.D.; Bedir, E.; Silva, B.; Khan, I.A. Frequency and timing of leaf removal affect growth and podophyllotoxin content of *Podophyllum peltatum* in full sun. *Planta Med.* **2006**, *72*, 824–829. [\[CrossRef\]](#)

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.