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Extraction and Purification of (E)-Resveratrol from the Bark of Conifer Species

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Abstract: (E)-Resveratrol is a naturally occurring polyphenolic compound in plants with a variety of widely studied health benefits. The bark of Northern American, Canadian, and Northern European conifer species, which is an underutilized by-product generated by forest industries, is a source of (E)-resveratrol, providing a potential value-added product for these industries. Bark may serve as a good alternative to the invasive plant Japanese knotweed (*Polygonum cuspidatum*), which currently is the leading commercial source of (E)-resveratrol. This work describes a method to extract and purify (E)-resveratrol from conifer bark with high yield and high purity and investigates the relationship between the amount of (E)-resveratrol and the total phenolic contents in the bark of common conifer species. In this work, barks of four conifer species were extracted and the total phenolic contents were determined by Folin–Cicoalteu's assay. The (E)-resveratrol content was determined by HPLC-MS. A purification method that utilizes solvent extraction and column chromatography was developed to isolate (E)-resveratrol in high yield from black spruce (*Picea mariana*) bark. The quantitative analysis of bark samples suggests the presence of (E)-resveratrol in black spruce (*Picea mariana*) and Norway spruce (*Picea abies*), in comparable amounts to Japanese knotweed. Based on HPLC-MS and HPLC-UV analyses, the purification method isolates the compound with a yield of 84% and purity of 99%. Hence, our method extracts and isolates (E)-resveratrol from conifer bark in high purity and high yield. The results do not support any correlation between the total phenolic content and the amount of (E)-resveratrol.

Keywords: (E)-resveratrol; extraction; purification; conifer bark

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1. Introduction

(E)-Resveratrol (5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol) is a stilbene derivative and a secondary metabolite produced in many terrestrial plants that include peanut, grape, and weeds such as Japanese knotweed [1]. The compound (Figure 1) has earned a reputation based on many research findings, providing evidence for its potential to benefit human health and wellbeing in a variety of different ways.

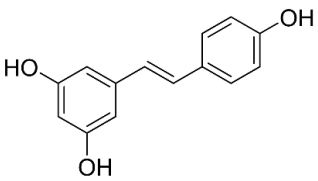

Figure 1. Chemical structure of (E)-resveratrol.

The polyphenolic chemical structure renders (E)-resveratrol the antioxidant activity for which it is well known [2]. In addition, many in vivo and in vitro studies provide supporting evidence for the ability of (E)-resveratrol to reduce obesity and hyperglycemia, and

to demonstrate anti-aging, anti-inflammatory, and anti-cancer properties [3–6]. Notably, a recent study has shown that (E)-resveratrol is capable of inducing neurogenesis, which suggests the potential of it to serve as a therapeutic agent for age-related neurodegenerative diseases [7]. These studies continue to secure a prominent place for (E)-resveratrol in pharmaceutical, food, and supplements industries, with a growing demand for the compound.

The production of (E)-resveratrol in plants is triggered by a wide variety of environmental factors such as pathogenic infections, damage by UV radiation to the tissues, and oxidative stress [8,9]. (E)-resveratrol in grapes and berries has found its way to red wine and is believed to be one of the reasons for the perceived health benefits of those beverages; however, some studies have shown that the residual enzymatic activity in them could decrease the resveratrol content by about 76% during storage [10]. Additionally, minute concentrations of this compound in many plants make them impractical sources of (E)-resveratrol [11]. Therefore, the current commercial source of this compound is a resveratrol-rich weed species, Japanese knotweed (*Polygonum cuspidatum*), which is also identified as an invasive plant [12,13]. Japanese knotweed root extracts account for many of the commercial preparations of (E)-resveratrol, and they possess a wide range of purity. Some studies suggest that Japanese knotweed root extracts, depending on their purity level, could contain compounds such as emodin, which confers laxative effects [14]. Additionally, the ability of Japanese knotweed to grow even in heavily polluted environments and heavy metal-contaminated soils, and absorb those contaminants through root tissues, pose further concerns on the safety of the knotweed root-based commercial products of (E)-resveratrol, depending on their purity [15,16].

The studies aimed at stilbene biosynthetic pathways that lead to the production of resveratrol and other polyphenolic compounds in spruces (*Picea* sp.) indicate that spruces inherently contain a large number of closely related polyphenolic compounds in addition to (E)-resveratrol. [17–19]. Further studies suggest that these compounds locate primarily towards the outer tissues and bark, where they provide defense against pathogenic infections [20].

In northern American states, Canada, and northern Europe, wood processing and forest bioproduct operations generate spruce bark in large quantities as waste. This waste is mostly used as a fuel to generate heat and energy for those mills. There is potential, however, to first extract high-value compounds such as (E)-resveratrol, and subsequently burn the bark for nearly full fuel value. In addition, it is informative to determine if the amount of (E)-resveratrol correlates with the total phenolic contents in the bark of commonly utilized spruces and to identify the species that have the greatest potential as sources of (E)-resveratrol.

Various extraction and purification methods for isolating (E)-resveratrol from several plant species including Japanese knotweed (*Polygonum cuspidatum*) [21,22], grapevine (*Vitis vinifera*) [23], peanut (*Arachis hypogaea*) [24], and some other medicinal herbs [25], are reported in the recent scientific literature. The unique and complex nature of the polyphenolic compounds profile in conifers renders the isolation of (E)-resveratrol from bark extracts challenging. The potential of the bark of black spruce (*Picea mariana*), one of the most important industrial conifer species in northern American forests, to serve as a source of (E)-resveratrol was suggested by a previous study [26] and by the research findings of our group. To the best of our knowledge, however, this study is the first to report a method aimed primarily at isolating (E)-resveratrol in high yield from conifer bark.

2. Materials and Methods

2.1. Plant Material

Healthy trees of commercially important conifer species including Norway spruce (*Picea abies*), black spruce (*Picea mariana*), white spruce (*Picea glauca*), and eastern white pine (*Pinus strobus*) were identified and harvested by the University of Maine research forest management group in 2008. For each tree, branches were removed, and the trunk was cut into 4-foot logs, which were then debarked by hand. Bark samples were air-dried for 72 h

and then ground into a powder using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). Powdered bark samples were stored in a freezer at −23 °C until further use.

2.2. Chemicals and Reagents

HPLC grade solvents that include chloroform, dichloromethane, diethyl ether, ethyl acetate, methanol, and toluene were purchased from Fisher Scientific (Waltham, MA, USA). The Folin and Ciocalteu's phenol reagent, Dichlorodimethylsilane (≥99.5%), formic acid (≥95%), sodium bicarbonate (≥99.7%), sodium carbonate (≥99.5%), 4-phenylphenol (≥97%), and phenolphthalein (≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The (+)-catechin (≥97%) standard was purchased from TCI America Inc. (Portland, OR, USA), and (E)-resveratrol standard was purchased from Mega Resveratrol (Danbury, CT, USA). Sephadex LH-20 column material was purchased from GE Healthcare Bio-Sciences (Pittsburgh, PA, USA). Cosmosil 75C18-OPN reversed-phase column material was purchased from Nacalai Inc. (San Diego, CA, USA). A Milli-Q water purification system (EMD Millipore, Billerica, MA, USA) was utilized for obtaining ultrapure water.

2.3. Moisture Content

The moisture contents of bark samples were determined by oven drying known masses of representative samples at 100 °C for 24 h and calculating the percent change in mass of each sample. It was incorporated in the calculations for the determination of total phenolic content and the amount of (E)-resveratrol in Norway spruce, black spruce, white spruce, and eastern white pine bark samples, which are reported as mass per dry weight of the bark.

2.4. Total Phenolic Assay

Each bark sample (3.0 g air-dried sample) was extracted using an accelerated solvent extractor (Dionex, Sunnyvale, CA, USA) with acetone/water (95/5, v/v) solvent mixture at 100 °C temperature and 1500 psi pressure for a total of 15 min (3 × 5 min static cycles). The resulting extract was divided into two equal portions and one portion was stored in the freezer until further use. The other portion was rotary evaporated to dryness and re-dissolved in 50 mL of water and extracted with hexane (3 × 35 mL) to remove hydrophobic extractives such as fatty acids and sterols from the aqueous layer. The remaining solution was extracted with ethyl acetate (3 × 50 mL) in order to isolate polyphenolic compounds. The ethyl acetate fraction was rotary evaporated to dryness and was re-dissolved in methanol (25.0 mL) before proceeding with the total phenolic assay.

The modified total phenolic assay used was first described by Singleton and Rossi [27]. The bark extract in methanol was diluted 2500-fold with the same solvent, and a 200.0 µL aliquot was mixed with 1.5 mL of Folin–Cicoalteu's test reagent, which was prepared by diluting the Folin and Ciocalteu's phenol reagent with water in a 1:10 ratio. After 5 min, 1.5 mL of aqueous sodium carbonate (7.5% w/v) was added to the mixture. The mixture was then allowed to stand for 1 h at room temperature before reading the absorbance at 725 nm using an HP diode array spectrophotometer (HP, Waldbronn, Germany). A standard curve was generated using (+)-catechin, and the total phenolic content in each bark sample was determined in catechin equivalents and expressed as mg per g dry bark. All analyses were performed in triplicate.

2.5. Quantification of (E)-Resveratrol

For the quantification of (E)-resveratrol, ethyl acetate extracts of each bark sample were obtained as described previously. Samples were rotary evaporated to dryness and reconstituted in methanol to obtain a 50-fold dilution, prior to the preparation for analysis by HPLC-MS (Agilent, Waldbronn, Germany). The (E)-resveratrol calibration curves were generated with the aid of phenolphthalein internal standard. Two analytical columns were used in succession for the separation (2 × Novapak C18, 3.9 × 150 mm). To obtain a good separation, a gradient solvent system was used at a flow rate of 0.3 mL/min, which

consisted of 1% formic acid (solvent A) and acetonitrile (solvent B): 0–5 min, 100% solvent A; 5–25 min 80% solvent A and 20% solvent B; 25–40 min, 100% solvent B; 40–45 min, 100% solvent A, with an additional 5 min post-run to equilibrate the column. The injection volume was maintained at 8 µL. All analyses were carried out in selective ion mode with negative ion polarity in the mass spectrometric detector. To detect single ion mass, an ESI source was used with nitrogen carrier gas (flow rate 12 L/min, nebulizer pressure 50 psi, drying gas temperature 350 °C). Capillary and fragmentation voltages were 3000 V and 70 V, respectively. Peak identity was confirmed by comparison of the retention time and fragmentation pattern to those of (E)-resveratrol standard. All determinations were performed in triplicate and expressed as µg per g dry bark.

2.6. Extraction and Purification of (E)-Resveratrol

Black spruce (*Picea mariana*) bark was the starting material for the extraction and purification of (E)-resveratrol. A total of 12.5 g of dry, ground bark was extracted for 12 h with 125 mL of diethyl ether in a Soxhlet apparatus, and the extraction was continued for an additional 12 h with another 125 mL of the same solvent. The two extracts were combined, and the total volume was reduced to 100 mL by rotary evaporation. The sample was washed with 5% (w/v) sodium bicarbonate solution (3 × 50 mL). The resulting organic phase (diethyl ether layer) was rotary evaporated to near dryness and re-dissolved in methanol in order to bring the final volume to 35.0 mL. An aliquot of 10.0 mL from the above sample was dried with 1 g of Sephadex LH-20 column material and loaded on a column (10.5 mm ID × 140 mm H) prepared with the same material in dichloromethane. The sample was eluted with dichloromethane until a fraction of 100 mL was collected, and then the mobile phase composition was changed to a mixture of dichloromethane and methanol in a 9:1 volume ratio. Column fractions of 25 mL were collected and analyzed by HPLC until (E)-resveratrol was eluted completely.

The fractions containing (E)-resveratrol were combined, rotary evaporated to a minimal volume, reconstituted in methanol, and the final volume was brought to 10.0 mL. A 3.0 mL aliquot of it was dried with 0.5 g of Cosmosil 75C18-OPN reversed-phase column material and loaded on a column (10.5 mm ID × 80 mm H) prepared with the same material in dichloromethane. The sample was eluted with a mobile phase comprising a mixture of dichloromethane and ethyl acetate in a 9:1 volume ratio, and 20 mL fractions were collected and analyzed by HPLC, until (E)-resveratrol eluted as a clean fraction. All glassware used in this step was silanized by soaking in 5% (v/v) dichlorodimethylsilane in toluene for 15 min, rinsing twice with toluene and once with methanol, and air-drying for 24 h.

Sample analysis and quantification at each step of the above purification process were conducted using an Agilent 1100 series HPLC with an automatic liquid sampler device and a variable wavelength (VW) detector (Agilent, Waldbronn, Germany). A Phenomenex Kinetex 5 µm C18 4.6 × 100 mm column (Phenomenex, Torrance, CA, USA) was used in the HPLC analysis, and the injection volume was maintained at 8 µL. For qualitative analysis, the detector wavelength was set to 306 nm and the mobile phase composition was set to change from 90% water and 10% acetonitrile to 60% water and 40% acetonitrile in 14 min followed by a hold time of 0.2 min and restoration of the starting composition in 1.3 min, at a flow rate of 1.5 mL/min. For the quantitative analysis, calibration curves of (E)-resveratrol were generated with the aid of 4-phenylphenol internal standard. All samples were analyzed at 306 nm for (E)-resveratrol and 260 nm for 4-phenylphenol. The mobile phase was composed of methanol:formic acid:water in 10:1:89 volume ratio (solvent A), and acetonitrile (solvent B). A gradient profile modified from Marshall et al. [28] was utilized in the HPLC: at 0 min, 100% solvent A; at 23 min, 54% solvent A and 46% solvent B; and at 38 min, 100% solvent A with a 2 min post-run with the same solvent. The VW detector wavelength was set to 306 nm for the first 27 min, 260 nm for the next 10 min, and 306 nm again for the remainder of the run. The total run time was 40 min, and the flow rate was 0.3 mL/min. All analyses were performed in triplicate and expressed as µg

per g dry bark. Table S1 in Supplementary Materials provides a detailed summary of the analytical results.

An Agilent 1100 series HPLC system equipped with a diode array detector (DAD) coupled to an Agilent 1100 series ion trap mass spectrometer with an electrospray ionization (ESI) source (Waldbronn, Germany) was used for further confirmation of the identity and purity of (E)-resveratrol isolated from the bark extracts. The same HPLC column and gradient profile described above were employed in the instrument, and the total ion chromatogram and mass spectrum of the sample were obtained and compared with the (E)-resveratrol standard in negative ion mode at 350 °C, 8.97 L/min drying gas flow, 49.9 psi nebulizer pressure, 3500 V capillary voltage, and 7.0 kV dynode voltage at 44.2 trap drive level.

3. Results and Discussion

3.1. Total Phenolic Content and (E)-Resveratrol in Bark Extracts

We investigated the total phenolic content in the bark of four common North American conifer species using Folin–Ciocalteu assay method and, as shown in Figure 2, the results from total phenolic assays indicate that white spruce bark has the highest amount of phenolic compounds in general.

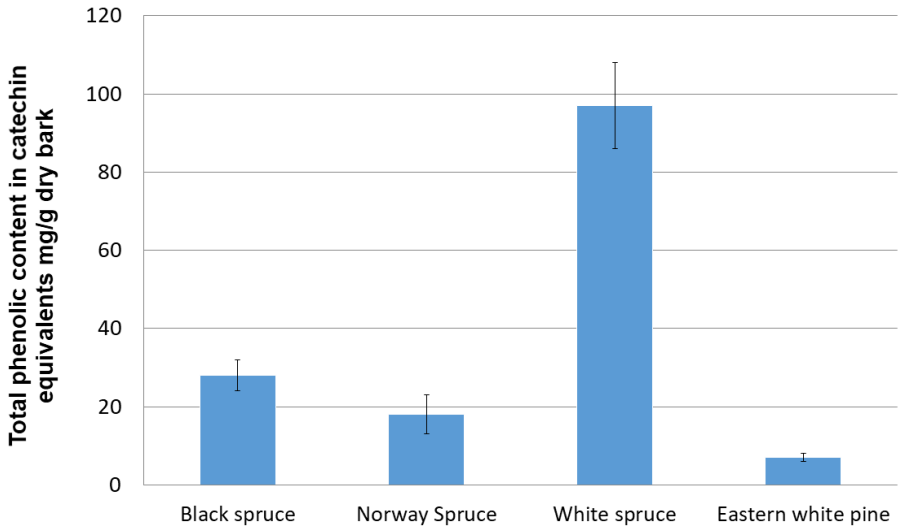

Figure 2. Total phenolic content in ethyl acetate soluble fractions of wood bark extracts.

The Folin–Ciocalteu assay is a widely used method to determine the total phenolic content of plant extracts. The reaction makes use of the ability of phenols to reduce the Folin–Ciocalteu reagent under basic conditions, which yields a colored product [29]. However, non-phenolic reducing agents such as sugars, vitamins, some amino acids, and inorganic salts interfere with this reaction, skewing the results [30,31]. Many of those interfering agents have a considerable solubility in water; hence, partitioning the plant extracts between water and a relatively non-polar solvent would minimize such interferences and increase the accuracy of the results. Therefore, the bark extracts were prepared to minimize the presence of those contaminants, as described in the experimental section.

Our findings from the quantitative analysis of ethyl acetate soluble fractions of the bark extracts show that while Norway spruce and black spruce have low total phenolic contents relative to white spruce, they contain high amounts of (E)-resveratrol (Figure 3). Hence, the results do not support a correlation between the total phenolic content and the content of (E)-resveratrol, which is not surprising due to the large number and types of phenolic compounds in the bark.

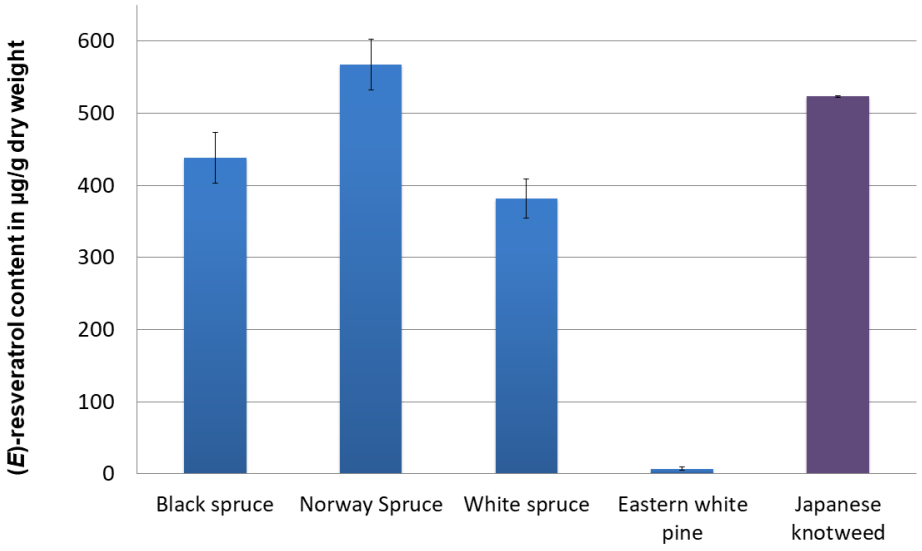

Figure 3. (E)-resveratrol content in ethyl acetate soluble fractions of wood bark extracts determined in this study, and freeze-dried Japanese knotweed (*Polygonum cuspidatum*) root as reported by Burns et al [11].

These results also indicate that spruces, especially black spruce (*Picea mariana*) and Norway spruce (*Picea abies*), have comparable amounts of (E)-resveratrol to freeze-dried Japanese knotweed (*Polygonum cuspidatum*) root extracts, and thus spruce bark is a potential commercial source for the isolation of (E)-resveratrol.

3.2. A Method for the Extraction and Purification of (E)-Resveratrol from Black Spruce Bark

The full extraction and purification procedure that we developed is shown in Figure 4. The crude diethyl ether extracts of the black spruce bark, after 5% sodium bicarbonate wash, revealed the presence of 332.9 ± 0.4 µg/g (E)-resveratrol upon HPLC analysis, and the final purified sample contained 279.9 ± 4.9 µg/g (E)-resveratrol. Hence, the overall recovery of (E)-resveratrol from this purification method is 84%.

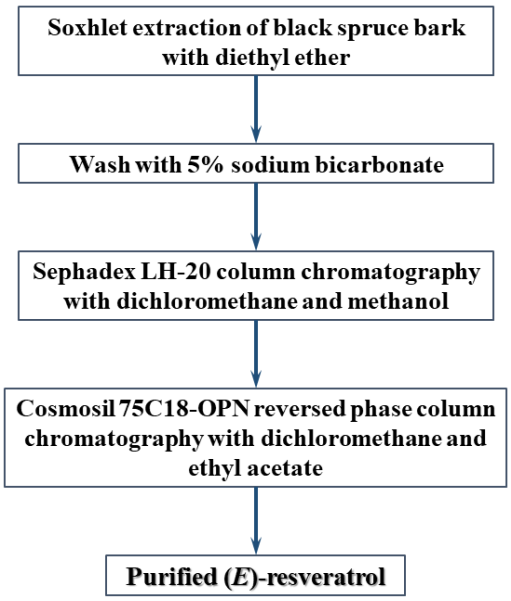

Figure 4. Flowchart of the purification procedure.

Confirmation of the purity and identity of the compound was achieved by HPLC-UV-MS analysis of the sample (Figure 5) against (E)-resveratrol standard. The purity observed

in HPLC-UV at 306 nm is 99%, and the retention time, molecular ion peak, and mass fragments matched with those of (E)-resveratrol standard. Figures S1–S3 in Supplementary Materials provide additional chromatographic data that confirm these results.

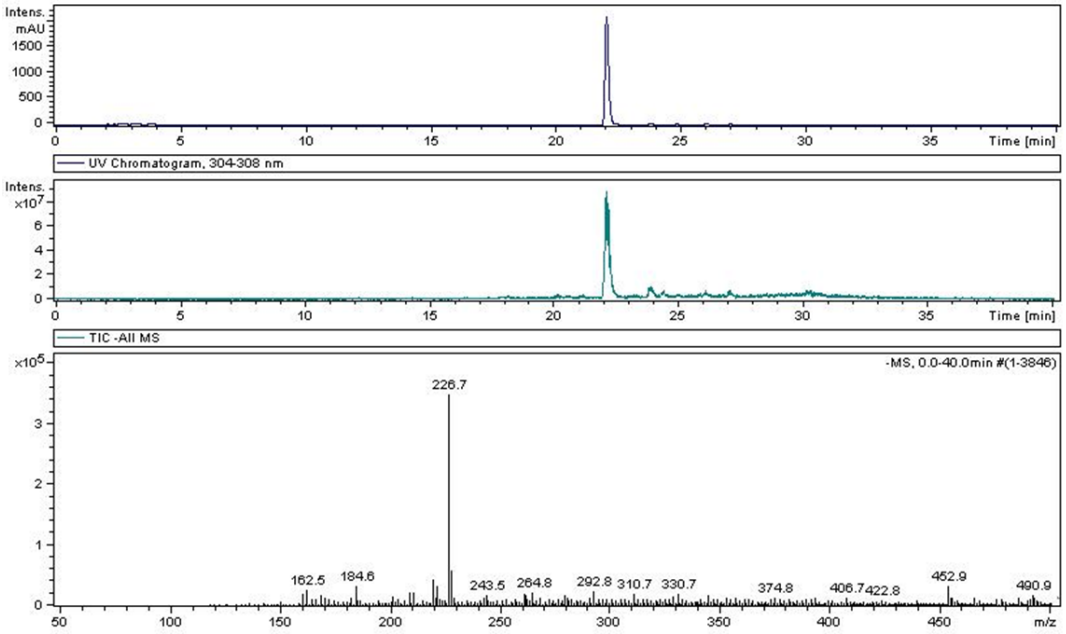

Figure 5. HPLC-UV chromatogram (at 306 nm) and HPLC-MS total ion chromatogram and averaged mass spectrum of the purified sample.

The process for extracting and purifying (E)-resveratrol from spruce bark proposed in our work combines novel approaches of chromatographic separation with some modified steps from recently published work on isolating (E)-resveratrol from Japanese knotweed (*Polygonum cuspidatum*) [21] and grapevines (*Vitis* sp.) [22]. The initial choice for the extraction solvent was methanol. However, reconstitution of the extract in diethyl ether to prepare it for the liquid–liquid extraction with aqueous sodium bicarbonate resulted in a sample loss of 32%, due to the rapid precipitation of insoluble compounds. Therefore, to minimize this loss, diethyl ether was used as the extraction solvent in the Soxhlet extractor. With its low boiling point of 35 °C, the heat required for the extraction was less, even though a higher volume of solvent and a longer extraction time were required due to the moderate solubility of (E)-resveratrol in diethyl ether. Extraction solvents similar to diethyl ether were investigated and discussed in recently published work aimed at obtaining resveratrol from Japanese knotweed rhizome [32].

The 5% sodium bicarbonate solution wash was employed to remove phenolic acids from the diethyl ether extract. The sample resulting from this step, upon reconstitution in methanol and HPLC analysis, revealed the presence of 332.9 ± 0.4 μg/g of (E)-resveratrol. In our experience, pretreatment techniques such as borate complexation extraction of vicinal diols [33,34], are good strategies to remove 1,2-diols from the bark extracts providing a passive purification, but the presence of a large number of non-vicinal diol-type polyphenolic compounds limits the ability of such pretreatments to bring about the desired purification on their own. In addition, passive purification techniques require chemicals in excess amounts and add several more steps to the purification process, causing further loss of yield.

Column chromatography with Sephadex LH-20 is discussed in the work of Güder et al. in relation to the isolation of resveratrol from fox grapes (*Vitis labrusca*) [23]. The Sephadex LH-20 column material is a cross-linked polysaccharide network with a large number of active sites for the interaction with analytes in complex mixtures, such as spruce bark extracts. Hence, careful selection of mobile phase compositions in the Sephadex LH-20

column chromatographic purification resulted in relatively clean fractions of (E)-resveratrol with a 319.6 ± 1.3 µg/g recovery. The fractions contained a few impurities, which were detected under the HPLC conditions mentioned previously, and they were observed overlapping with the Z-isomer of resveratrol (present in the sample in minute amounts). Studies on stilbene biosynthetic pathways in spruces have no supporting evidence to conclude that Z-isomer of resveratrol is synthesized alongside the E-isomer. Hence, the detection of (Z)- resveratrol in bark extracts could be attributed to the photoinduced isomerization of (E)-resveratrol [35,36], in situ, or during analysis. Extreme care was taken in the sample preparation and purification process in this study to minimize E to Z isomerization.

To purify the eluate from the Sephadex LH-20 column chromatography, a chromatographic step with Cosmosil 75C18-OPN reversed-phase column material was introduced. The chromatographic conditions in this step were defined to simulate the separation occurring in the HPLC column. The results indicated a clean fraction of (E)-resveratrol with a 279.9 ± 4.9 µg/g recovery. Due to the non-polar nature of the column material and the mobile phase, glassware used in this chromatographic step required surface deactivation by silanization to improve the yield.

4. Conclusions

Studies on stilbene biosynthesis pathways in spruces suggest a high complexity in their polyphenolic compound profiles. Hence, developing an extraction and purification process aimed at isolating (E)-resveratrol from the bark is a challenging research problem. We anticipate, however, that the black spruce (*Picea mariana*) bark used in this study to extract, purify, and isolate (E)-resveratrol represents a comparable complexity in its polyphenolic content which should make this purification method applicable to other types of spruces as well.

The method described here is capable of isolating (E)-resveratrol from black spruce bark in high purity (99%, as observed in HPLC-MS and HPLC-DAD at 306, 286, and 260 nm) and high yield (279.9 ± 4.9 µg per 1 g of dry bark, 84% recovery). Commercial application of this method would lead to a new use for waste conifer bark and provide forest products industries in the northern United States, Canada, and northern Europe with a high-value bioproduct to enhance their revenue. Additionally, isolation of high purity (E)-resveratrol from a renewable, non-invasive natural resource has environmental, ecological, and potential medicinal benefits.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr10040647/s1, Figure S1: Final purified product HPLC chromatograms at common detector wavelengths (306, 286, and 260 nm) for polyphenolic compounds, Figure S2: HPLC chromatograms at 306 nm from the major steps of the spruce bark purification process, Figure S3: LCMS TIC and mass spectrum of purified BS sample in comparison to resveratrol standard, Table S1: Summary of quantitative analysis of (E)-resveratrol content at each step of the purification process.

Author Contributions: Conceptualization, B.J.W.C. and R.C.F.J.; methodology, R.L. and P.S.P.; investigation, R.L. and P.S.P.; writing—original draft preparation, P.S.P.; writing—review and editing, B.J.W.C.; supervision, B.J.W.C., A.D.M. and R.C.F.J. All authors have read and agreed to the published version of the manuscript.

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