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Original Article

Atefeh Saadabadi, Abrar Ahmed, Annika I. Smeds and Patrik C. Eklund*

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Abstract: Stilbenes are plant polyphenols that have shown beneficial pharmacological activities in a variety of diseases. The considerable amount of stilbene glucosides in spruce inner bark encouraged us to develop a straightforward and simple method of extraction with high recovery and yield. Stilbene glucosides from fresh inner bark of Norway spruce were extracted in one simple step with acetone at 20 °C. After three weeks of soaking in acetone, the extracts were dried and the composition was determined by GC-FID using a short and a long column (HP-5) and GC-MS (HP-1). The amount of the extracted compounds was also compared with a similar extract from air dried inner bark samples. The extracts from the fresh sample contained 30–50% of stilbene glucosides and the average yield [0.185 g extract/g bark] was as good as or slightly better than in previously reported works. However, no drying, milling, or sequential extractions with different solvents in elevated temperatures were needed. Moreover, this study revealed that the drying process can decrease the amount of extractable stilbenes significantly. Therefore, this method can be considered as an alternative for preparative isolation of stilbene glucosides, especially isorhapontin and astringin from inner bark of Norway spruce.

Keywords: extraction; inner bark; Norway spruce; *picea abies*; stilbene glucosides.

***Corresponding author: Patrik C. Eklund**, Laboratory of Molecular Science and Engineering, Faculty of Science and Engineering, Åbo Akademi University, Biskopsgatan 8, Åbo 20500, Turku, Finland, E-mail: patrik.c.eklund@abo.fi. https://orcid.org/0000-0003-3040-5116
Atefeh Saadabadi, Laboratory of Molecular Science and Engineering and Pharmaceutical Sciences Laboratory, Faculty of Science and Engineering, Åbo Akademi University, Turku, Finland
Abrar Ahmed, Punjab University College of Pharmacy, University of the Punjab, Lahore, Pakistan
Annika I. Smeds, Laboratory of Natural Materials Technology, Faculty of Science and Engineering, Åbo Akademi University, Turku, Finland

1 Introduction

Inner bark of Norway spruce (*Picea abies L. H. karst*) is a valuable source of bioactive components, especially of stilbene glucosides such as isorhapontin, astringin and piceid (resveratrol glucoside) (Figure 1) (Krogell et al. 2012). In recent years, stilbenoids have received enormous attention due to their broad range of biological activities, ranging from antimicrobial, antifungal and anti-inflammatory activities to neuroprotective, cardioprotective and anticancer effects. Since their exact molecular mechanism still remains unknown in most health conditions, they constitute a hot topic for further investigation of biological activities (Akinwumi et al. 2018). Wood-based stilbenoid aglycones such as pinosylvin and pinoslylvin monomethyl ether (pinosylvin MME) have been shown to exhibit anti-inflammatory effects, possibly by inhibition of the transient receptor potential ankyrin 1 (TRPA1)ion channel (Nalli et al. 2016). As the stilbene glucosides are deglycosylated to the aglycones in the liver, they can be considered a source of stilbenes with higher solubility and the same biological activities (Henry-Vitrac et al. 2006). Therefore, stilbene glucosides from the inner bark of Norway spruce may find applications as bioactive health-promoting compounds, which encouraged us to develop an easy method for extracting and preparing these compounds.

During the last decades, much research has been carried out on the extraction, purification, characterization and utilization of *P. abies* bark constituents. In all previous isolation methods, the inner bark, after separation from the outer bark, was first freeze-dried or air-dried, then milled and subjected to one of the following extraction techniques: Soxhlet extraction (Bukhanko et al. 2020; Burčová et al. 2018), supercritical fluid extraction (SFE) (Bukhanko et al. 2020; Co et al. 2012), accelerated solvent extraction (ASE) (Krogell et al. 2012; Välimaa et al. 2020), pressurized fluid extraction (PFE) (Co et al. 2012) or conventional solvent extraction (SLE) (Co et al. 2012). Generally, at the first step of isolation, lipophilic extractives such as resin acids, fatty acids and terpenoids were separated with a

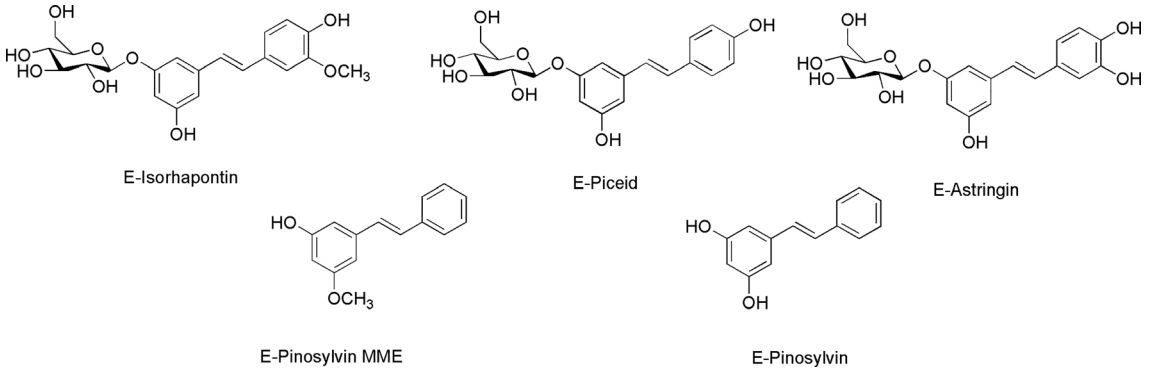

Figure 1: The structures of stilbenoids in Norway spruce inner bark.

nonpolar organic solvent in diverse temperatures, and in the second step, a hydrophilic solvent or a polar solvent mixed with water was used for the extraction of hydrophilic extractives such as stilbene glucosides, tannins and sugars (Co et al. 2012; Zhang and Gellerstedt 2008). In 2008, Zhang and Gellerstedt separated lipophilic compounds from air-dried inner bark in two steps, first with petroleum ether and then with dichloromethane. Eventually, an extract enriched in stilbene glucosides and tannins was isolated with acetone at room temperature (Zhang and Gellerstedt 2008). In another study, Krogell et al. (2012) separated 10.6% stilbene glucosides with a combination of acetone/water (9:1, v/v) at 100 °C, after the extraction of lipophilic components with hot hexane (90 °C) by ASE. In (2012), Le Normand et al. extracted less than 9% stilbene glucosides mixed with sugars from whole fresh bark with acetone. Furthermore, 2.7–4.8% stilbene glucosides were successfully isolated from a freeze-dried sample with acetone-water (95:5, v/v) by Jyske et al. (2014).

During bark processing, especially in large-scale, the content of stilbene glucosides is known to decrease drastically over time. This phenomenon is not fully understood, but it may be due to hydrolysis, leaching, oxidation, degradation and/or polymerization in exposure to UV light, heat and air (Jyske et al. 2020; Silva et al. 2014).

In order to optimize the recovery and yield of stilbene glucosides from the inner bark of Norway spruce for this study, the aim was to find a fast, easy, and low-cost extraction method that would be applicable in gram as well as kilogram scale. Thus, stilbene glucosides from the fresh inner bark of Norway spruce were isolated in one step with acetone, without drying, at an ambient temperature (20 °C). The yield and the composition of the extract was compared with the results obtained by previously reported methods. Also, the effect of drying was studied to assess the feasibility and efficiency of this fast and simple approach.

2 Materials and methods

2.1 Chemicals and equipment

Methanol (MeOH) and dichloromethane (DCM) were purchased from Sigma-Aldrich (St.Louis, MO, USA) in analytical grade (99.9%). Silica gel 60 (0.040–0.063 mm) and TLC Silica gel 60 F254 from Merck (Darmstadt, Germany), and Sephadex® LH-20 from Fluka (Buchs, Switzerland), were used for column chromatography. Technical acetone and pyridine were obtained from VWR (Fontenay-sous-Bois, France). Acetone (HPLC grade) and Dimethylsulfoxide-d_6 (99.8%) for diluting the extracts prior to GC and NMR analysis were from Sigma-Aldrich (Steinheim, Germany) and Eurisotop (St.Aubin, France), respectively. The silylation reagents, bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were purchased from Acros Organics (Geel, Belgium) and Aldrich Chemicals (St.Louis, MO, USA), respectively. All internal standards were obtained from Sigma-Aldrich (St.Louis, MO, USA) except betulinol that was isolated from birch bark and purified at the Laboratory of Natural Materials Technology at the Åbo Akademi University. The GC-FID was Clarus 500 (short column: 6–7 m × 0.530 mm i.d., 0.15 μm), Autosystem XL (long column: 25 m × 0.20 mm i.d., 0.11 μm) (Perkin Elmer Inc., Waltham, MA, USA). The GC-MS instrument was a Hewlett Packard G1530AGC coupled to a 5973 MSD (Hewlett Packard, Palo Alto, CA, USA) with a similar 25 m column as mentioned above (Smeds et al. 2012).

2.2 Bark material and isolation process

Four samples of Norway spruce almost in same size and age (25–30 years old) were felled at two different locations (Southwestern Finland) at around 10 km distance from each other. Two of them (A and B) were collected in the Kimito island (60°10′N, 22°36′ E) and the other two (C and D) were retrieved from Sandö (60°13′N, 22°28′ E) in June 2020 (on the same date). Each sample was collected 2–4 m from ground, after a complete separation of the inner bark from the outer bark and xylem with a blade based on visual comparison. The samples were chopped in small pieces (approximately 1 × 2 cm) with a blade, then a 500 g portion was immediately transferred to an amber bottle containing 2 L of technical acetone. After three weeks of soaking in the acetone at the ambient temperature (20 °C), the filtrate was evaporated first with a rotary evaporator at 40 °C and then kept in a vacuum oven

for three days at 40 °C. For comparison, 100 g of each bark sample was dried in a dark place at ambient temperature (20 °C) for three weeks and in the vacuum oven (40 °C) for one more week. The weight loss was assessed gravimetrically for determination of the water content. The air-dried bark was subjected to the same isolation process as mentioned earlier for the fresh samples.

2.3 Purification of stilbene glucosides

Two grams of the acetone extract was loaded on a Sephadex LH-20 column and eluted with methanol. Eventually, two main fractions were separated: the first one contained isorhapontin and the second one consisted of astringin. For further purification, each fraction was subjected to a normal phase column chromatography (silica gel 60) and eluted with DCM and methanol as a solvent system starting with 100% DCM and gradually decreasing to proportions of 10:1 and 5:1 [DCM/MeOH]. The verification of the structures was made by comparing their NMR data with the previously published literature (Mulat et al. 2014).

2.4 GC and GC-MS analysis of the extracts

An exact amount (2–3 mg) of dry acetone extracts from each sample was dissolved in 1.5 ml acetone in GC vials. A volume corresponding to 0.15 mg of the dry extract was placed into a test tube and 2 ml of an internal standard (IS) solution containing 0.02 mg each of the pure compounds (>95%) n-heneicosanoic acid, betulinol, cholesteryl heptadecanoate, and 1,3-dipalmitoyl-2-oleyl glycerol was added. The solvent was evaporated to dryness. For the calculations, n-heneicosanoic acid (21:0) was used as IS for all compounds eluting before the lignans, and betulinol was used for lignans and stilbenes except the *E*-isomers of pinosylvin and pinosylvin MME. Cholesteryl heptadecanoate was used as IS for steryl esters and diglycerides, and 1,3-dipalmitoyl-2-oleyl glycerol for the triglycerides. After evaporation, silylation reagent, 0.12 ml of pyridine/BSTFA/TMCS [1:4:1], was added to the samples, which were heated at 70 °C in an oven for 30 min. The samples were analyzed by GC-FID using a short column, two long columns in parallel (HP-1 and HP-5) and GC-MS (HP-1). The GC-FID and GC-MS conditions have been described in detail in Smeds et al. (2019). The identification of individual compounds was done primarily by comparing the characteristic mass spectra of each compound with those in databases (NIST12/Wiley11th and the database developed at the Laboratory of Wood and Paper Chemistry at the Åbo Akademi University). In addition, numerous components were identified by comparison to authentic reference compounds available in the laboratory.

3 Results and discussion

3.1 Total extraction yield

The acetone extracts (Supplementary Figure S1) were evaporated and dried to give a yellow powder as residue. The dry product was weighed to determine the total amount of extract per sample (500 g fresh inner bark). The amount of extract from highest to lowest was observed in samples C (54.5 g), A (48.8 g), D (45.1 g) and B (34.3 g). The average yield of dry acetone extract from the fresh inner bark was 9.1%. The water content of the fresh inner bark samples was in average 53%, resulting in an average yield of 18.5% extract based on dry weight. Further, it was observed that the extraction yield from the dried inner bark samples was much lower than that from the fresh bark (Table 1).

3.2 Chemical content and composition of the extracts

The chemical content and composition of the acetone extracts from both fresh and dried inner bark were analyzed by GC-MS (Supplementary Figure S2) and GC-FID (Supplementary Figure S3). Although the chemical composition of all acetone extracts from fresh inner bark resembled each other, significant variation in the content was observed. For instance, the concentration of fructose and other monosaccharides in samples A and B (collected from the same place), were twice as high as those in C and D (Table 2). Furthermore, an isomer of astringin (here called "astringin2") was detected only in sample A. Sample A contained also a higher concentration of *Z*-isorhapontin than samples B, C and D. Monoterpenes were only identified in samples A and D. On the other hand, the content of steryl esters in sample A was found to be lower than in the other samples, while sample D contained the highest amount of triglycerides. The amount of stilbene glucosides in sample C was remarkably higher than in the other samples. The amount of *E*-piceid and *E*-isorhapontin were about two times and *E*-astringin about three times higher in sample C than in the other samples. Moreover, in sample C *E*-astringin was present at a higher concentration than *E*-isorhapontin, whereas the ratio was opposite or equal in

Table 1: The extraction yield in grams (g) from 500 g fresh and 100 g dried inner bark and the percentage of water content in each sample.

Sample	Amount of extracts from 500 g fresh bark	% Extract from fresh sample*	% Extract from dried sample*	Water content (%)
A	48.8 (9.8%)	20.1	8.5	51.6
B	34.3 (6.9%)	15.4	4.1	55.4
C	54.5 (10.9%)	20.4	9.9	53.3
D	45.1 (9.0%)	18.3	6.7	50.6

*Calculated on dry inner bark material.

the other extract samples. Although all trees were almost of the same size and age, the bark of tree C had a noticeable mechanically induced injury. A possible explanation for the higher amount of stilbene glucosides in sample C could be the defense mechanism of the tree in response to injury or fungal infection (Latva-Mäenpää et al. 2013).

Overall, the extraction yield of stilbene glucosides from the fresh samples of inner bark was estimated to be 5.6, 3.7, 11.1 and 4.9% for samples A, B, C and D, respectively, with the average yield of 6.3% that is comparable or even better than the yields reported in previous studies, except for the study by Krogell et al. (2012) that reported 10.6%.

The chemical content, yield and composition of the extracts isolated from the dried bark samples were assessed to evaluate the effect of drying process and air exposure on the yield of stilbenes, specifically the glucosides. The percentage of fructose, other monosaccharides, pinitol, sucrose (except in extract B), pinoresinol and steryl esters in the extracts from dry samples declined remarkably, while the amount of fatty acids and catechin (except in extract B) significantly increased in comparison to the extracts from the fresh samples. However, the increase of fatty acids was insignificant in sample C. The dried extracts contained gallocatechin and monopalmitin (except in extract A) that were not detectable in the fresh bark extracts. Triglycerides could not be detected in the dry samples. It should be noted that the amount of water present will affect the composition of the extract since dry acetone does not extract carbohydrates to the same extent as a water/acetone mixture, however, the extraction of stilbene glucosides should not be significantly affected.

The analysis also indicated that the amount of pinosylvin, pinosylvin MME, *Z*-astringin and *Z*-isorhapontin decreased significantly in the dry samples (Table 2). Although the abundance of other stilbene glucosides (*E*-isorhapontin, *E*-astringin and *E*-piceid) remained almost without notable changes, the total concentration of

Table 2: Extract composition from the four fresh and dry inner bark samples, concentration (mg g^{-1} extract).

Sample compounds	A		B		C		D	
	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Fructose	39.9	0.9	30.3	0.8	15.0	1.2	13.8	nd
Other monosaccharides	51.7	nd	43	1.5	20.2	nd	18.2	nd
Pinitol	12.2	2.5	9.1	5.4	0.0	4.6	12	6.61
Quinic acid	1.3	nd	1.3	nd	nd	nd	nd	nd
Fatty acids	4.5	18.5	37	13.7	3.08	3.4	2.9	33.8
Monopalmitin	nd	nd	nd	1.4	nd	1.5	nd	8.2
Monoterpenes	6.8	0.8	nd	nd	nd	nd	4.2	6.9
Resin acids	40.6	38.4	30.4	31.4	31.4	27.9	33.9	55.4
Sucrose	23.3	6.8	6.6	9.4	15.2	7.3	18.5	7.2
Catechin	6.5	36.5	79	48.1	7.03	28.7	10	36.5
Gallocatechin	nd	4.3	nd	7.5	nd	2.6	nd	8.1
Pinoresinol (+NTG)	52.5	3.9	59.2	3.5	67.2	3.5	32.8	3.7
Stilbenes								
Pinosylvin (E+Z)	27.7	nd	26.8	1.6	20.4	nd	16.9	nd
E-Pinosylvin MME	47.2	3.6	45.2	1.9	35.2	2.3	28	nd
Z-Isorhapontin	40.1	5.6	22.7	5.7	20.2	4	27.7	4.3
Z-Astringin	22.4	2.7	16.8	3.8	24.1	5.3	17.4	2.7
Astringin2	5.3	4.7	nd	nd	nd	nd	nd	nd
E-Piceid	17.4	17.8	14.9	15.4	34.0	21.9	13.3	12.5
E-Astringin	84.1	71.9	94.8	91.8	291	167	99.4	100
E-Isorhapontin	107	120	92	107	177	112	107	116
Stilbenes, total	352	226	313	227	602	312	310	235
Unidentified on long column	87.6	74.1	80.1	76.4	135	60.9	90	128
Diglycerides	8.4	8.5	11.1	8.3	7.4	5.8	10.3	7.10
Steryl esters	9.2	3.7	16.6	4.06	24.4	4.4	27.1	4.7
Triglycerides	14.5	nd	18	nd	6.2	nd	23.3	nd
Total	634	425	630	438	943	464	607	541
% non-GC eluting	37	57	37	56	5.7	54	39	46

nd, not detected; NTG, nortrachelogenin.

stilbenes generally dropped about 14% on average in the acetone extracts after the drying process (Figure 2). Although the hypothesis about the negative effect of drying on the yield was proved, the mechanism behind it remains unknown. It may be due to chemical changes in the structures of the components or a physical condition in the wooden matrix leading to interference in the extraction process. According to these results, skipping the drying process not only saves time and energy but it can also enhance the yield of stilbenes.

3.3 Isolation and purification

The acetone extracts A and C (2 g) from the fresh samples were chosen for purification with column chromatography. After pre-purification using a Sephadex LH-20 column, sample A provided a fraction of 450 mg and sample C a fraction of 354 mg containing *E*-isorhapontin (impure). Likewise, a fraction of 350 mg from sample A and a fraction of 877 mg from sample C, containing *E*-astringin (impure) were separated. Finally, purification of *E*-isorhapontin on a silica column using DCM/MeOH [10:1] yielded 380 mg (from sample A) and 252 mg (from sample C), while purification of *E*-astringin with DCM/MeOH [5:1] yielded 292 and 678 mg from sample A and C, respectively. The isolated yields from sample A were 19% for *E*-isorhapontin and 14.6% for *E*-astringin. From sample C, the yields were 12.6% for *E*-isorhapontin and 33.9% for *E*-astringin. The amount of the isolated compounds indicated that the extracts contained 30–50% of stilbene glucosides. The purity of the isolated compounds was over 95% as determined by ^{1}H and ^{13}C NMR spectra (Supplementary Figures S4-S8, Supplementary Tables S1 and S2) and GC-MS (Supplementary Figures S9-S10). The isolated yield calculated on dry inner bark material (sample A) was 2.9 and 3.8% for *E*-astringin and *E*-isorhapontin, respectively. For sample C, the isolated yield was 6.9% for *E*-astringin and 2.6% for *E*-isorhapontin. The amount of *E*-isorhapontin was higher than *E*-astringin for sample A while this ratio was opposite in sample C, which was in accordance with the GC-MS and GC-FID results.

The results pointed out that this method is well suitable for laboratory scale preparation of the said compounds. However, no limitation in upscaling of this simple method can be foreseen, even at an industrial scale, as the whole bark can also be used in the extraction process. The composition of acetone extract from outer bark (Krogell et al. 2012) should not interfere with the extraction and the yield of stilbene glucosides from the inner bark, although a

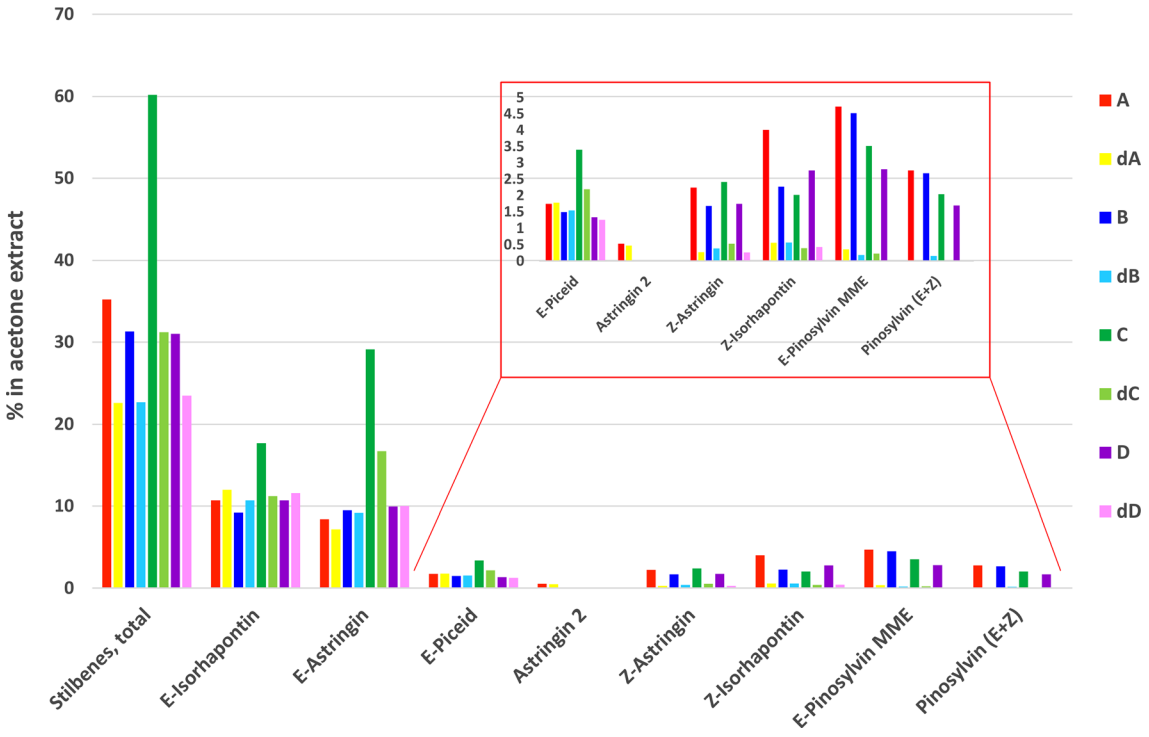

Figure 2: The yield of stilbenes in the acetone extracts for fresh samples A (red), B (blue), C (green), D (purple) and dried samples dA (yellow), dB (light blue), dC (light green), dD (pink).

slightly different extract will be obtained when the whole bark is used.

4 Conclusions

The composition of Norway spruce inner bark has been investigated in several earlier studies, and stilbene glucosides have been extracted in various yields from 1.4% (Gabaston et al. 2017) to 10.6% (Krogell et al. 2012). Since previous studies were not entirely focused on stilbene glucosides, elaborate techniques using a combination of solvents even in high temperatures to isolate various components from dried bark, were employed. In this work, stilbene glucosides were successfully and selectively extracted in high yields (6.3%) from fresh spruce inner bark with acetone in a simple extraction process. The extraction time was long to ensure a complete extraction and high yield. However, this time can be reduced to a few days without a severe decrease in yield and recovery, which makes this method feasible also for large-scale extractions. As assumed and demonstrated, a drying process does have a detrimental effect on the outcome of the extraction of stilbenes and significantly reduces their amount. Interestingly, this effect was more evident for the *Z*-isomers. In conclusion, this approach can be considered as a straightforward, easy, and scalable laboratory method for the isolation of stilbene glucosides from the fresh inner bark of Norway spruce.

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