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Determination of the Extractive Content of Western Redcedar by Liquid–Liquid Fractionation and Gas Chromatography–Mass Spectrometry Analysis

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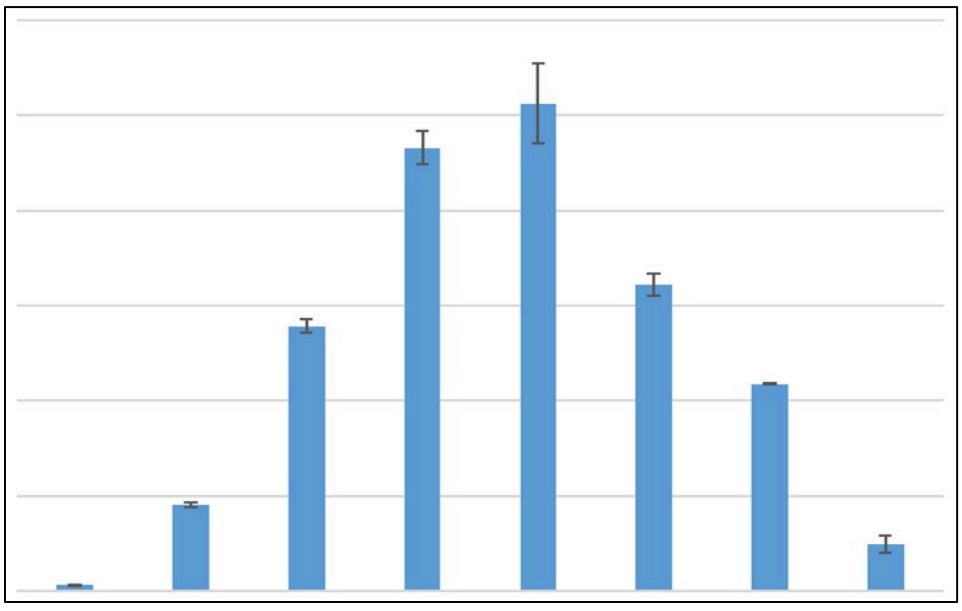

Abstract

Many durable wood species contain extractives with biologically active chemical components that have potential to be used for the development of pharmaceuticals. For this study, extractive compounds from western redcedar (*Thuja plicata*) were separated by their relative polarities using liquid–liquid fractionation and characterized through gas chromatography–mass spectroscopy (GC–MS). Fractionation was successful at separating the components of western redcedar in a consistent manner, and GC–MS was able to identify the presence of various terpenoid compounds, primarily nezukone and thujaplicins. Most of the compounds were in low concentrations, and broad peaks were observed, which indicates that more resolving power is needed to further characterize the compounds in the fractions. Four compounds were identified in low concentration, including alpha and gamma eudesmols, which are known for having pharmaceutical effects on tumor activity. These compounds will be further investigated and the fractions characterized utilizing two-dimensional GC time of flight mass spectrometry.

Keywords: western redcedar, liquid–liquid fractionation, extractives, durability, *Thuja plicata*

Contents

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Introduction

Cedars are well-known trees and are used in many applications, including siding, furniture, boatbuilding, and greenhouses, to name a few. These trees are known as naturally durable softwood tree species because of their extractive content in the wood. Cedar trees are vital to sustainable societies and economic systems (PlantSnap 2022).

Western redcedar (*Thuja plicata*) (WRC) is naturally resistant to decay and insect damage, so no chemical treatment of its lumber is needed for typical applications (naturally:wood 2022). WRC can be used in a wide variety of interior or exterior building applications because it is a resilient and versatile species. WRC resilience is due its heartwood extracts. Twenty-one extractives have been identified in WRC, and many others remain to be characterized (Morris and Stirlin 2012). WRC extractive compounds include terpenoids, thujaplicins, and assorted lignans (Morris and Stirlin 2012). Many of these compounds have been found to be linked to the durability of WRC, particularly two of the identified lignans (plicatic acid and plicatin) that have exhibited resistance to wood decay (Stirling and Morris 2016). Thujaplicins have been shown to have strong antifungal capabilities, although there is not a strong correlation between the thujaplicin content of western redcedars and their durability (Stirling and Morris 2016). Morris and Stirling (2012) identified an unknown (compound B) that appeared to have decay resistance. Compounds that improve the durability of wood are biologically active and therefore can potentially be used as building blocks for new pharmaceutical products (Moore et al. 2015, Murzin et al. 2007).

Gas chromatography–mass spectrometry (GC–MS) is an analytical method used to characterize components within western redcedar extractives. Liquid–liquid fractionation (LLF) was used to separate the WRC extractive compounds base on their polarity. The fractions of the WRC extractives will assist with preventing coelution and column saturation of concentrated compounds, which can interfere with the identification. The fractionation methods could also provide insight into how potentially valuable compounds from WRC extractives could be efficiently isolated. This study utilizes simple mixtures of two cost-effective and readily available solvents, acetone and hexane, to separate WRC extractives for characterization using GC–MS.

Experimental Methods

Sample Preparation

Western redcedar bark samples were cut and Wiley milled to 40 mesh. All samples were isolated by Soxhlet extraction using a 9:1 acetone:water wash. Samples were then dried using a rotary evaporator and stored in a freezer.

Liquid–Liquid Fractionation

Approximately 100 mg of sample was weighed and fractionated using the hexane:acetone ratios listed in Table 1. Each of the solvent mixtures was added to a 3-mL vial containing extractives, sonicated, and then mixed using a vortex. Samples were left to settle, and then the supernatant was decanted into a 7-mL vial. The extractives were washed with 9 mL of each solvent mixture. The fractionated samples were then dried under nitrogen to a consistent weight and then air dried overnight. Three replicates were performed over consecutive days.

Table 1—Solvent ratios used for extraction

Vial label	Hexane:acetone solvent ratio (v:v)
B	10:90
C	20:80
D	30:70
E	45:55
F	60:40
G	80:20
H	100:0

Gas Chromatography–Mass Spectrometry

Analysis of the extractives was performed using an HP 5890 Series II Plus Gas Chromatograph (HP Inc., Palo Alto, California) instrument equipped with an HP-5 capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness). The column temperature was initially 60 °C for 1 min, then gradually increased to 300 °C at 10 °C/min and kept there for 3 min. Extractives were dissolved in 500 to 1,000 µL of 1:1 (v/v) acetone:methanol, depending on weights, then pipetted into 1,000-µL GC vials. Vials of the dissolved sample were then injected automatically into the instrument.

Results and Discussion

Visual Observations

As the polarity of solvents increased, the sample progressed from being a brown viscous solution to a very granular red-brown powder by solvent F (60:40 hexane:acetone). The samples gradually darkened, starting as a clear yellow and acquiring an orange-red tint by solvent G (80:20 hexane:acetone) (Fig. 1). There appeared to be no correlation between the amount of analyte dissolved in each solvent and the darkness of the supernatant, implying that the more polar fractions contain more darkly colored compounds. The analytes dissolved in vials G and H likely consisted primarily of lignans, which are thought to be the primary contributor to the color of western redcedar (Tasnim et al. 2020). Lignans are phenolic, so their appearance in the most polar solution is to be expected. The GC–MS spectra for samples G and H had no major peaks identified, suggesting that the lignans were not volatilized due to their high molecular weights.

Polarity Fractionation

Figure 2 shows that some sample was left in vial A. The residue mass left in vial A ranged from 0.3 to 0.7 mg. The goal of the LLF was to distribute all extractives proportionately among the different solvent solutions. The bar graph in Figure 2 shows that the sample was distributed in various amounts as the polarity of the solutions changed. The amounts of sample in vials B to E increased as the polarity of the solutions increased, with a strong affinity for solvent solutions in vials D and E. (Vial E contained only 3% more sample than vial D.) However, as the solvent becomes more polar than vial E, the mass of the sample decreased. Figure 2 shows the distribution of the sample weight percentages fractionated into each vial. In trial 1 (blue dashed line), some sample was lost during the transfer into vial F. The error bars represent the calculation for trials 1 and 2. Moore et al. (2015) separated extracts using hexane:chloroform (5:1) as the solvent mixture and collected 97% polar and 3% nonpolar samples. One of the goals of this study was to improve the sample distribution for WRC extractives. Hexane:acetone solvent mixtures were successful at fractionating the WRC extractives sample.

Gas Chromatography–Mass Spectrometry

All fractions were characterized using GC–MS. Vials B, C, D, and E sample mass increased as the polarity of the solution mixture increased. We expected to identify major compounds in each of these samples. Fraction B contained several long-chain alkanes. Most extractives had aliphatic long chains. Fraction B also contained traces of oleic and n-hexadecanoic (palmitic) acid. One of the least concentrated compounds was nopol, a terpene that has antimicrobial properties (Pillai and Endalkachew 2004, Luts et al. 1964), which was observed in fractions B and D. It is used in the synthesis of pesticides, soaps, detergents, and other household products. It is a sweet, balsamic, and citrus-tasting compound. Another least concentrated peak corresponded to thujaplicins, a known component of WRC extractives (Maclean and Murakami 1966), which were observed in vial C (Fig. 3A). Hinokitols are potent ingredients that have anti-inflammatory, antioxidant, antibacterial, antifungal, and antimelanogenic properties. They target inflammatory redness and blemishes seen in rosacea and acne (Luts et al. 1964). Nezukone was observed in vials B to D (Morris and Stirling 2012). These are also both notably cycloheptenol compounds, suggesting that this may be a common motif in this type of tree species.

The peaks present in the chromatograms for the most polar fractions (F to H) were mostly broad, with just a few sharp peaks with low concentration. Guaiacylacetone was the only compound identified in the polar fractions (Fig. 3C). This compound could come from the degradation of lignan compounds (Tasmim et al. 2020). Although the results were consistent the literature by showing the presence of nezukone and thujaplicin, other known compounds, such as plicatic acid and plicatin, were not observed. Any plicatic acid could have been changed into matairesinol and then converted to dihyroxy thujaplicatin. The biosynthesis of these chemicals has not been fully elucidated (Stirling and Morris 2016). The GC–MS spectra were not able to capture compounds with low concentrations or low volatility. Both nezukone and thujaplicins are known for being biologically active and could potentially be important for pharmaceutical applications (Johansson et al. 2000).

Four compounds were identified that were not previously mentioned in the literature. α-eudesmol; γ-eudesmol; 1,3,5-cycloheptatriene, 7,7-dimethyl-; 3-oxabicyclo[5.3.0]decan-2-one, 9-methylene-, cis- are listed in Tables 2 and 3. The

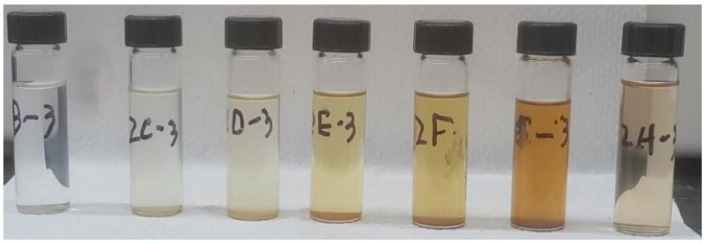


Figure 1—Supernatant containing dissolved analyte following a replicate of liquid–liquid fractionation. Vial A, containing residual sample, is not pictured.

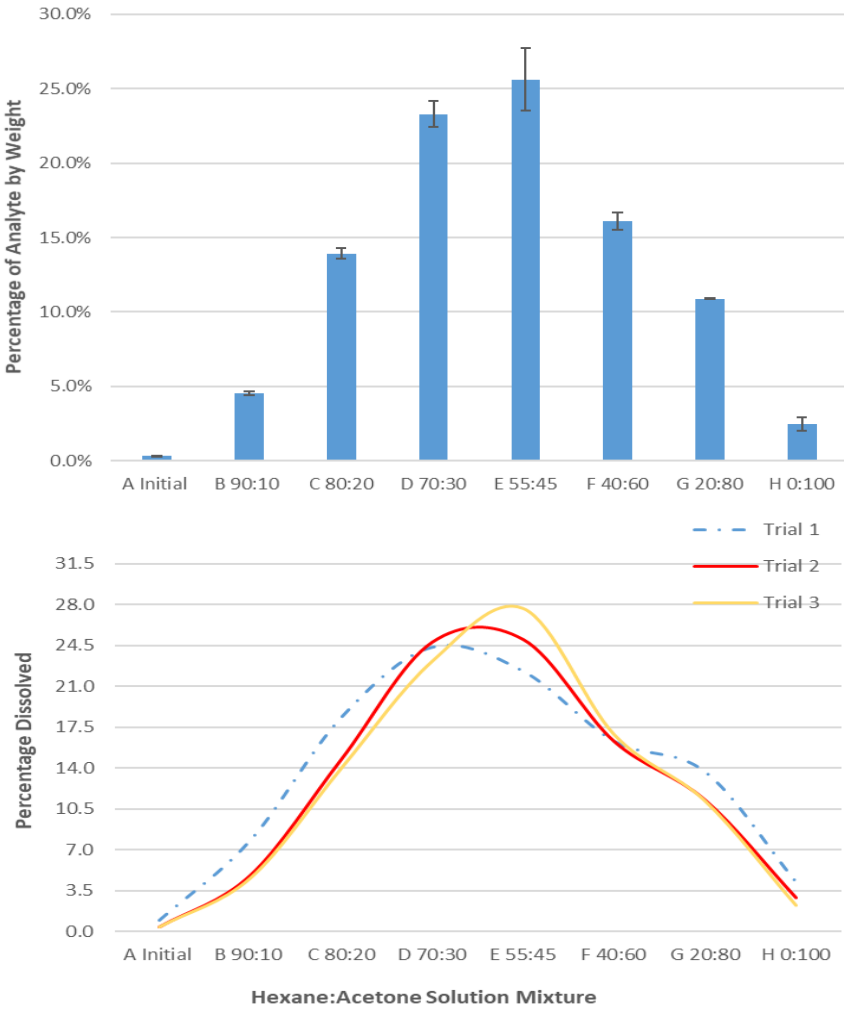


Figure 2—Dissolved distribution of analytes in each solution mixture. Standard error bars are shown in the histogram for 95% confidence intervals.

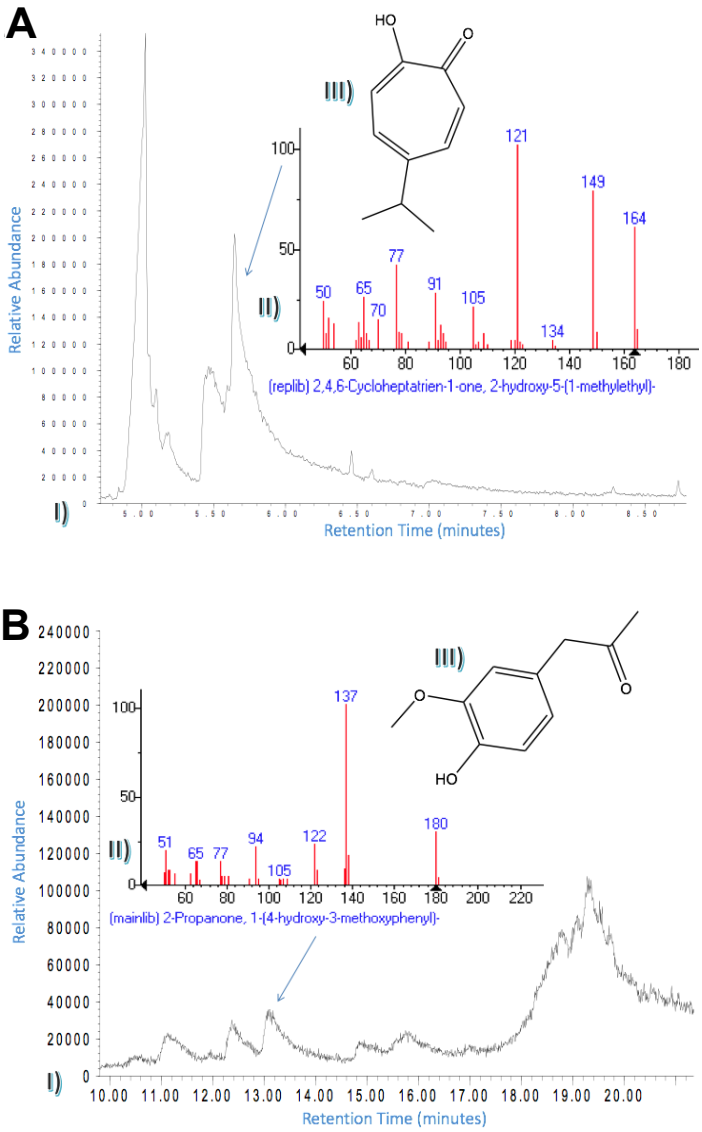


Figure 3—GC–MS data: (I) chromatograms, (II) spectra, and (III) chemical structures similar to (A) thujaplicin and (B) guaiacylacetone. Compounds were identified in solvents B & C and structures were generated using ChemDraw Ultra 7.0.

Table 2—Western redcedar compounds identified from each vial fractionation using GC–MS

Solvent	Compound name	Retention time (min)
B	Nopol	11.111
	3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, cis-	11.204
	Nezukone	11.355
	1,3,5-Cycloheptatriene, 7,7-dimethyl-	11.478
	α-Eudesmol	13.390
	Palmitic acid	16.784
	Heneicosane	17.927
	Oleic acid	18.596
	Docosane	18.797
	Tricosane	19.660
	Pentacosane	21.256
C	3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, cis-	11.116
	Myrtenoic acid, ethyl ester	11.116
	Nezukone (major)	11.303
	1,3,5-Cycloheptatriene, 7,7-dimethyl-	11.526
	β-Thujaplicin	11.835
	γ-Thujaplicin	11.835
	γ-Eudesmol	13.388
D	Nopol	10.895
	3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, cis-	10.960
	Nezukone	11.715
E	Guaiacyllacetone	13.093
	2-Methoxy-4(methoxymethyl) Phenol	18.778
F	Guaiacylacetone	13.365
G	Guaiacylacetone	13.509

Table 3—The structure of the compounds identified from western redcedar extractives fractionation

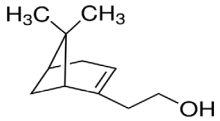
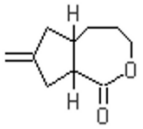
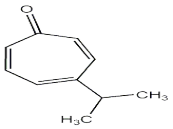
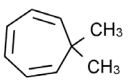
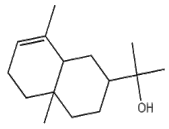
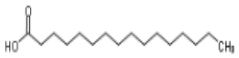
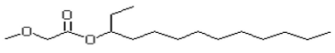
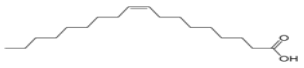
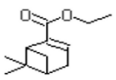
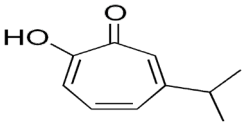
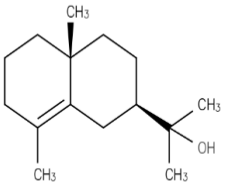
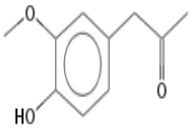
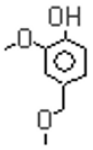
Vial	Compound name	Molecular formula
BD	Nopol	
BCD	3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, cis-	
BCD	Nezukone	
BC	1,3,5-Cycloheptatriene, 7,7-dimethyl-	

Table 3—The structure of the compounds identified from western redcedar extractives fractionation—continued

B	α-Eudesmol	
B	Palmitic acid	
B	Methoxyacetic acid, 3 tridecyl ester	
B	Heneicosane	$C_{21}H_{44}$
B	Oleic acid	
B	Docosane	$C_{22}H_{46}$
B	Tricosane	$C_{23}H_{48}$
B	Pentacosane	$C_{25}H_{52}$
C	Myrtenoic acid, ethyl ester	
C	Thujaplicin	
C	γ-Eudesmol	
EFG	Guaiacyllacetone	
E	2-Methoxy-4(methoxymethyl) phenol	

tables list the retention times, chemical structures, and the compounds identified in each vial. These tables include known compounds found in the literature. The alpha and gamma eudesmols were identified in vials C and D, respectively. The eudesmols are sesquiterpenoid alcohols that have pharmaceutical effects. The alpha, beta, and gamma eudesmols display cytotoxicity against tumor cell lines.

The broad and low concentrations of the compounds identified will be further investigated. We were able to fractionate based on the polarity of the compounds, which assists with identifying the compounds in the different fractions using GC–MS. Further characterization utilizing two-dimensional gas chromatography time of flight mass spectrometry (GC×GC TOFMS) with more resolving power will assist with the identification of the trace and low-concentration compounds in the fractions.

Conclusion

LLF using various ratios of hexane:acetone solutions distributed the samples into different fractions. It was observed that most of the samples were distributed among the solvent solutions with close to 50% of the two solvents. Plicatic and plicaten were not identified; however, the biosynthesis pathway of these chemicals is not well known in the literature. Four compounds not listed in the literature were identified. The alpha and gamma eudesmols compounds were two of the four identified. These compounds are known for having antitumor activity and low systemic toxicity (Britto et al. 2012). Most of the compounds identified were in low concentrations. Characterization via GC–MS corroborated literature in identifying known compounds, such as nezukone and the thujaplicins. However, there were four fractions (vials F–H) that contained only one identified compound. Further investigation will utilize two-dimensional GC time of flight mass spectroscopy (GC×GC TOFMS), which is known for its resolving power and ability to identify trace compounds in low concentration.

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