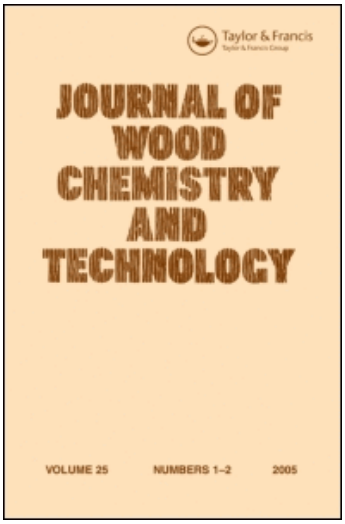

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New Method to Isolate Gamma-Thujaplicin from Western Red Cedar (*Thuja plicata* Donn.)

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Abstract: Western red cedar (WRC) (*Thuja plicata* Donn.) is one of the most durable softwoods in North America, and this durability has largely been attributed to its tropolone content. Further studies on factors contributing to the durability of this species require isolation of substantial quantities of the active components. Wood meal from the heartwood butt of a WRC tree was extracted with hexane for 24 hours. The thujaplicin mixture was obtained by chloroform extraction of the alkali solution adjusted to a pH of 6.5. With chemical conversion and regeneration, the selected compound, "gamma-thujaplicin," was isolated from the thujaplicin mixture and characterized by Fourier transform infra-red (FTIR) spectroscopy, High Performance Liquid Chromatography (HPLC), Mass Spectroscopy (MS), ^{1}H-nuclear magnetic resonance (NMR), Gas Chromatography–Mass Spectroscopy (GC–MS), a color test, and comparison with standard gamma-thujaplicin. Relative purity reached up to 99%. Additionally, a by-product, nezukone, could be also isolated from the hexane extractives.

Keywords: Gamma-thujaplicin, GC–MS, HPLC, identification, isolation, MS, western red cedar

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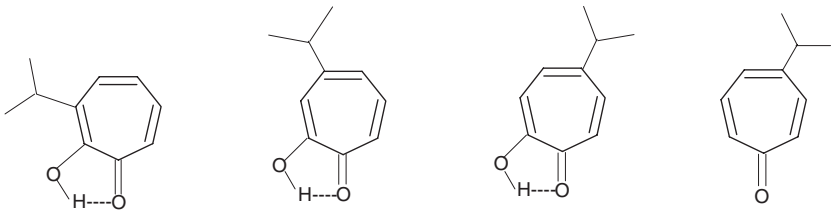
INTRODUCTION

Investigation of the natural fungicides found in the heartwood of many species has been attracting much attention from researchers.[1–28] Western red cedar (WRC; *Thuja plicata* Donn.) is one of most durable softwoods in North America. This durability is often attributed to the presence of thujaplicins, which are toxic to many decay fungi. Gamma-thujaplicin (2-hydroxy-5-isopropyl- 2,4,6-cycloheptatrien-1-one) is one of three isomeric isopropyltropolones found in the heartwood of WRC.

WRC is a massive tree, which grows up to 60 m high and attains a diameter of up to 6 m. It has small dark-green, shiny scale-like leaves and grows well on moist soils and sphagnum peat bogs.[29] WRC is found on the Pacific coast from southeast Alaska to Oregon and in the wet belt in the interior of British Columbia, northern Idaho, and western Montana.[30] In Canada, WRC's growth is limited to British Columbia.[31]

WRC has many potential uses in industries where decay resistance is desirable.[32] It is especially valuable for shingles, poles, posts, and exterior siding. Generally, most WRC goes into lumber, shakes, and shingles, and small amounts as furnish for kraft paper.[33] One of the most important properties of WRC, and probably the most variable, is its natural durability. The antifungal property of the outer heartwood of WRC has been responsible for its use as a natural fungicide. Additionally, the thujaplicins have gained commercial attention. For example, they have been used in many fungicidal preparations,[34,35] as well as in hair and skin lotions.

The natural durability of WRC has been attributed to the presence of beta- and gamma-thujaplicin. The current belief is that the thujaplicins and lignans are part of an elaborate defensive arsenal that has evolved to protect the heartwood from invasion by rotting fungi and bacteria.[36] Further studies on the role of beta-thujaplicin and gamma-thujaplicin in WRC durability are needed. Their molecular structures are shown in Scheme 1.



Scheme 1. Molecular structures of thujaplicins.

The chemistry of WRC has been reviewed by Barton and MacDonald.[32] The major substances in the volatile fraction of the extractives (and their percent in red cedar heartwood) are alpha-, beta-, and gamma-thujaplicin (0.51%),

beta-dolabrin (0.0003%), beta-thujaplicinol (0.07%), thujic acid (0.08%), and methyl thujate (0.17%). The basic chemical structure of these substances is identified as a seven-membered carbon ring with various isopropyl, ketone, and hydroxyl groups at different ring positions. The proportion of each chemical constituent depends on the methods of extraction and the part of the tree extracted.

The objective of this study was to develop a simple and efficient method to extract, isolate, and identify selected gamma-thujaplicin in the outer heartwood of WRC with hexane. The ultimate goal was to isolate and purify substantial quantities of all major extractives to facilitate further biological testing.

EXPERIMENTAL

Materials

The raw material was the second-growth of WRC (<100 yrs) in British Columbia, Canada. Based on the previous work of Daniels and colleagues[27,36] the selected outer butt heartwood of second-growth of WRC (Figure 1) was cut from 30 or 25 growth rings from the sapwood/heartwood interface. The selected material was made into very small thin slices and dried about 1 week at room temperature. The slices were ground to wood meal passing through a 5-mm sieve. The moisture content at this stage was 6.23% by oven drying (105 ± 2°C).

Extraction Method

Five batches of 800-g wood meal were each extracted for 24 hours with the same 10 liters of n-hexane leave large volume Soxhlet Extractor with a 12 L flask. Fresh hexane was added after each batch to maintain constant volume after exchanging extracted material for new wood meal. About 47-g (wet basis, without solvent) extractives was obtained from 800-g (OD basis) of wood flour. A total of 235.2-g (wet basis, without solvent) of hexane extractives were obtained from a total of 4000-g of wood flour.

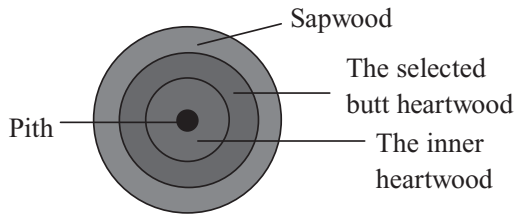


Figure 1. Schematic of WRC disk.

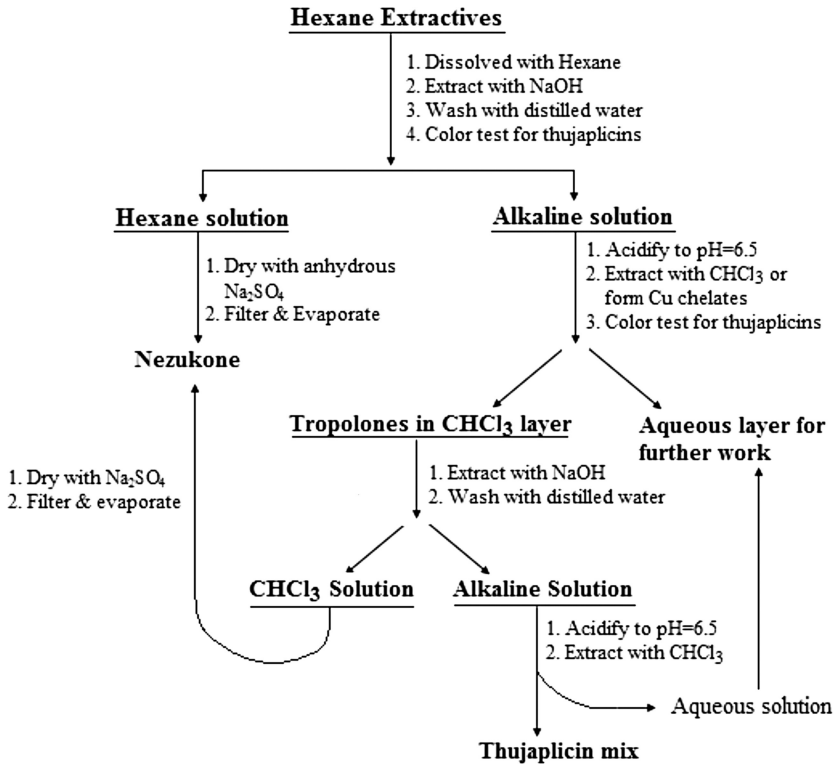


Figure 2. The scheme of separating thujaplicin mixture from hexane extractives.

Separation of Thujaplicin Mixture from the Hexane Extractives

The separation of the thujaplicin mixture used an improvement on the work of Su.[37] For hexane extractives, the improved method is illustrated in Figure 2, except for the change in extraction solvent. 7.10-g of hexane extractives (wet basis) was dissolved in 200-mL hexane and transferred to a separatory funnel, and extracted with a 2% sodium hydroxide solution (100 mL × 2), and washed once with 100-mL of distilled water. This isolated hexane layer was enriched with nezukone. The absence of thujaplicins in this hexane layer was shown by the absence of a positive red color with the addition of 5% $FeCl_3$. The hexane solution was dried over anhydrous sodium sulfate, filtered again and the solvent removed under reduced pressure to leave nezukone (1.84-g).The alkaline solution was acidified to pH 6.5 with 10% sulfuric acid, and extracted with chloroform (100 mL × 4). The chloroform layer was separated, dried, and filtered. The chloroform solution was enriched in thujaplicins as indicated by a positive red color test with 5% $FeCl_3$. The solvent was removed to yield a

tropolone mixture (2.29-g). 2.29-g of tropolone mixture dissolved in 120 mL chloroform was extracted with sodium hydroxide again (50 mL × 2). 0.56-g nezukone was obtained from the chloroform layer; its relative concentration was 92.0%. 0.66-g thujaplicin mix was obtained from alkaline solution, which was acidified and extracted with chloroform (50 mL × 4) again.

Separation and Purification of Gamma-Thujaplicin

The method shown in Figure 3 was designed for the isolation and purification of gamma thujaplicin.

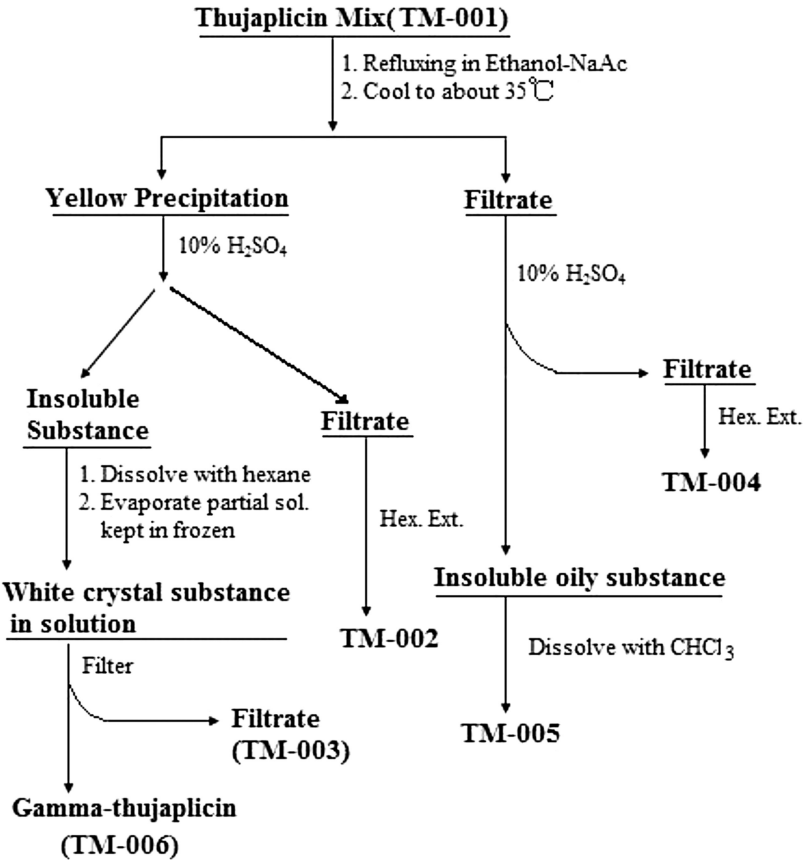


Figure 3. Isolation and purification of gamma-thujaplicin.

Identification of the Isolated Gamma-Thujaplicin

High Performance Liquid Chromatography (HPLC) analyses were previously reported by Daniels and Russell.[27]

MICROMASS® Quattro micro™ API Mass Spectroscopy (MS): Ionization mode: ES-; Data type: Enhanced Mass; Function type: Scan; Mass range: 30 to 500

^{1}H-nuclear magnetic resonance (NMR): ^{1}H-NMR spectra was taken on AV400 Bruker NMR spectrometer (made in Switzerland).

Gas Chromatography–Mass Spectroscopy (GC–MS): The isolated compound was determined by GC–MS. For GC–MS analysis, a 6890GC-5973MSD instrument was used (Agilent, USA). Capillary column was DB-17 (30 m × 0.25 mm, 0.25 μm film thickness). Carrier gas was He, 1 mL/min. Split injection (1.0 μL) with a ratio of 1:20 was employed. Injector temperature was 260°C, oven temperature was programmed starting at 40°C, then increased at 20°C per min to 160°C, need for 6 min; and then increased at 5°C per min to 240°C and need for 2 min. Temperature of the MS transfer line was 280°C, EI with 70 eV. Temperature of MS ion source was 230°C. Scan range was 15–260 amu. Identifications were based on a comparison of the GC retention times and EI (electron impact) ionization mass spectra with those in our database.

Fourier transform infra-red (FTIR) spectroscopy: Gamma-thujaplicin (GT-S, 500 μg/mL in ethanol), beta-thujaplicin (BT-S, 500 μg/mL in ethanol), and the selected compound (#1, 1015 μg/ml in ethanol) were scanned from 4000 cm^{-1} to 400 cm^{-1} using a FTIR spectrometer. Two or three drops of samples were added to the center of the IR card. After the solvent evaporating, the IR card was put on the holder and was scanned. The background of solvent was scanned in advance. The assignments for the major peaks of two standards and the selected compound were listed in Table 1.

RESULTS AND DISCUSSION

Extraction Methods Comparison

Extraction methods for WRC include hot water extraction, steam distillation, organic solvent extractions in acetone, benzene, ethanol, or chloroform, and supercritical carbon dioxide extraction. Different extraction methods obtain different yields with varying extractive makeup. Chang and colleagues[20–23] and Yen et al.[28] in Taiwan soaked the heartwood of 4 kinds of different species (*Taiwania cryptomerioides, Cinnamomum osmophloeum, Cryptomeria japonica*, *C. macrolepis* var. *formosana*) in 95% ethanol for 7 days, then used a different solvent such as n-hexane, ethyl acetate, or ethanol to further fractionate the extractives with Liquid Chromatography (LC), Thin Layer Chromatography (TLC), and HPLC. Another isolation method from Su[37] was also tried in the tropolone mixture. Five percent cupric acetate solution and hydrogen sulfide

Table 1. The peaks assignments of FTIR spectra for three samples with disposable IR Card

Wavenumber of BT Standard (cm^{-1})	Wavenumber of GT Standard (cm^{-1})	Wavenumber of the isolated compound (#1) (cm^{-1})	Assignment of peak
3383	3382	3420	OH stretching
2961			
2917	2918	2911	CH stretching
2848	2868	2846	
2360	2360	2348	=C−H stretching
	2108	2142	
1652	1646	1645	C=O stretching
1061	1061	1061	C−O stretching or OH deformation(bending) vibration
913	924	920	
726	737	729	C−C deformation(bending) vibration
667	667	681	

gas (H_2S) were used. The stink smell in the operation was not good to health. Sometimes this isolation did not work, because the black precipitation (CuS) was very difficult to filter, easy to be brought into the filtrate and resulted in influencing the filtrate color and HPLC analysis. Generally, these isolation methods are effective, but some of the procedures are difficult, and TLC isolation can be tedious. However, the used method in this study is simple and efficient, without using TLC and LC. Furthermore, the isolation and purification are easy to control. The isolated "gamma-thujaplicin" was the final product. During the repetition, the lowest relative purity was 86.0%; the highest could reach up to 99.1%. The solution temperature must be strictly controlled in the isolation. This kind of isolation method has potential value in the batch production.

HPLC Analyses of Gamma-Thujaplicin

According to the HPLC results, the compositions of thujaplicin mixtures were almost the same. Thus, the thujaplicin mixture solution was combined and evaporated to yield 3.64-g of the thujaplicin mixture (TM-001). The thujaplicin mixture (3.64-g approximate 49% gamma-thujaplicin, 31% beta-thujaplicin with 13% unknown compound as well as traces of nezukone, thujaplicatin ME, and beta-thujaplicinol), and anhydrous sodium acetate (2.0-g, it was dried for 1 h above 120°C in an oven before use) were refluxed in sodium-acetate-saturated ethanol (about 37 mL) for 40 min. The solution was naturally cooled

to about 35°C and the precipitated yellow sodium salt and excess sodium acetate obtained by filtering. The precipitated salts were enriched with respect to gamma-thujaplicin, while the filtrate was enriched with beta-thujaplicin.

The precipitated yellow salts were transferred to a dry beaker to which 50 mL of 10% H_2SO_4 solution was added. The insoluble yellow-white granules were obtained by filtration (0.59-g). This precipitate was dissolved in 25 mL of hexane; some white crystal and a little red brown blot appeared (sometimes refrigeration was needed to induce cystallization). By filtering, a white crystal was obtained (0.15-g) and dissolved in 15 mL of hexane (TM-006). After the analysis of HPLC, TM-006 had the same retention time as the gamma-thujaplicin standard and only one major peak appeared in the TM-006; the relative concentrations of gamma-thujaplicin in TM-006 were 99.06%. If the purity of gamma-thujaplicin is not required to be very high (more than 80%), only using chloroform to dissolve the white crystal is enough. The HPLC of the separated nezukone and gamma-thujaplicin is shown in Figure 4 compared with that of the hexane extractives.

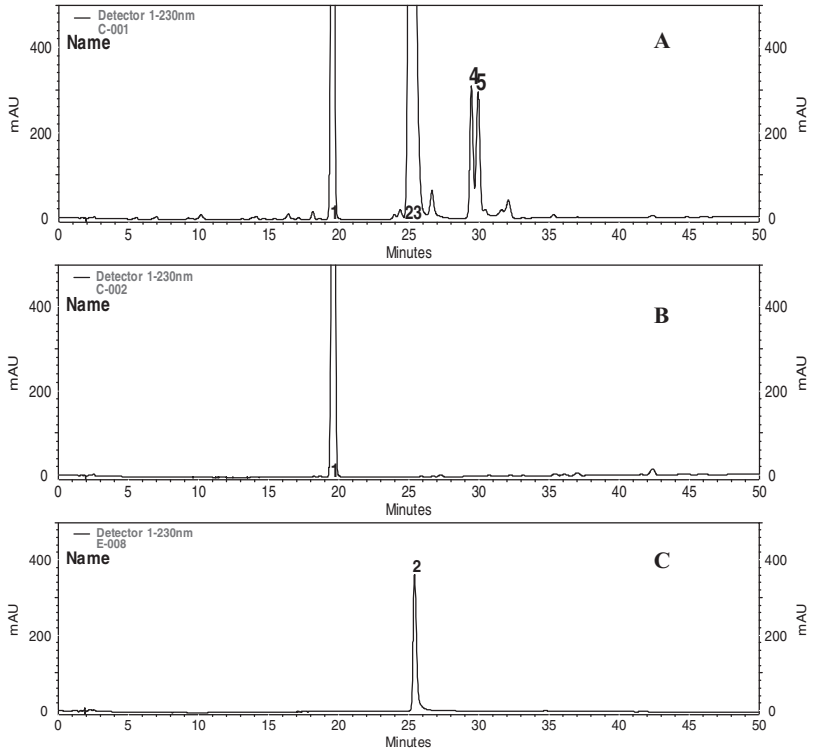
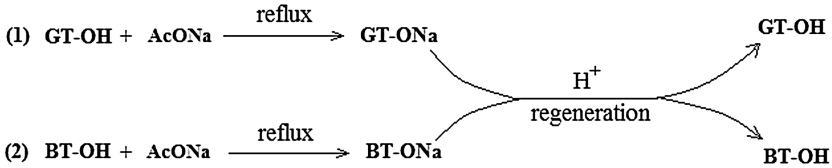


Figure 4. The separated nezukone and gamma-thujaplicin from the hexane extractives (A:1-Nezukone, 27%; 2-Gamma-thujaplicin, 30%; 3-Beta-thujaplicin,18%; 4-Thujic acid, 6%; 5-Unknown, 7%; B: 1-Nezukone, 92%; C: 2-Gamma-thujaplicin, 99%).

The isolated component #1, from TM-006, was tested by HPLC. The result showed that the isolated component had the same retention time as the gamma-thujaplicin standard (GT-S) under the same analyzing method. Its purity was 99.06% by HPLC. This indicated that the isolated component was very likely gamma-thujaplicin.

The Mechanism of Conversion and Regeneration for Gamma-Thujaplicin

The formal structures of gamma-thujaplicin and beta-thujaplicin are listed above; they can be simplified as GT-OH and BT-OH, respectively. When the thujaplicin mixtures are refluxing with excessive anhydrous sodium acetate (AcONa) and anhydrous ethanol, chemical reactions occur (Scheme 2):



Scheme 2. Conversion and regeneration of beta-, and gamma-thujaplicin.

The melting points of gamma-thujaplicin and beta-thujaplicin are 80°C and 52°C, respectively.[38] The solubility of their sodium salts is different with the temperature going down. GT-ONa (yellow) should firstly precipitate compare with BT-ONa after cooling the mix solution naturally. Furthermore, sodium-acetate saturated ethanol could enhance the yellow precipitation to some extent. At about 35°C, the yellow precipitation and excess sodium acetate were obtained by filtering. By regeneration with 10% H_2SO_4, the insoluble substance was obtained and dissolved with n-hexane. White crystals appeared in the hexane solution after cooling (sometimes in a refrigerator). The white crystal was the selected compound.

The Color Test of the Selected Compound

A part of the selected compound (isolated gamma-thujaplicin) was dissolved with chloroform in a small test tube, and then a few drops of 5% ferric chloride solution were added to chloroform layer. After shaking, the positive red color immediately appeared in the chloroform layer. This color test indicated that the selected compound was a tropolone, which contained a carbonyl and an adjacent hydroxyl on its seven-member unsaturated carbon ring.[38,39] However, this color test is a method of characterizing tropolone; it cannot differentiate alpha-, beta-, and gamma-thujaplicin.

The Analysis for Gamma-Thujaplicin with MS and ^{1}H-NMR

On the basis of previous analytical methods developed by FPInnovations–Forintek Division, for the standard substance, the isolated thujaplicin was believed to be gamma-thujaplicin. In order to further identify it, the beta-thujaplicin standard (BT-S, 500 μg/mL in ethanol), gamma-thujaplicin standard (GT-S, 500 μg/mL in ethanol), and the selected compound (#1, 1015 μg/mL in ethanol) from WRC were injected into a mass spectrometer under the same condition, respectively. The molecule fraction spectrums of the three samples are listed in Figure 5.

Figure 5 shows that there are obvious differences between BT-S and GT-S in m/z of 107.5 and 135.0. Furthermore, there is no the m/z of 107.5 and 135.0 in the GT-S MS and #1 MS. Comparing GT-S with #1, it is observed that the major molecule fraction peaks of #1 sample were very like those of GT-S. The m/z of 239.7, 240.0 and 240.1 appears respectively in the corresponding MS of #1, GT-S and BT-S. But the m/z of 239.7 shows the strongest signal peak in molecule fraction peak of #1. For this stronger signal peak, one of the reasons may be due to the distance or steric effects of space rearrangement of molecule fraction. In general, their molecule fraction peaks between GT-S MS and #1 MS are almost the same.

^{1}H-NMR (400MHz, $CDCl_3$, δ, ppm) spectra of GT-S and #1 were scanned and analyzed. The spectrum assignment of GT-S was 1.26 (6H, m, $-CH_3(\times 2)$), 1.39(1H, m, H-6), 2.10(1H,m, H-7), 2.90(1H,m, $-$CH), 3.71(1H, m, H-4), 4.33(1H, s, $-$OH), 7.26 (1H, m, H-3). However, the assignment of #1 was 1.26 (6H, m, $-CH_3(\times 2)$), 1.39(1H, m, H-6), 1.99(1H,m, H-7), 2.92(1H,m, $-$CH), 3.71(1H, m, H-4), 4.70(1H, s, $-$OH), 7.26 (1H, m, H-3). By comparison, the chemical shifts of GT-S and the isolated #1 were almost same. This provides further evidence indicating that selected compound (#1) is gamma-thujaplicin.

The Analysis of Gamma-Thujaplicin with GC-MS

According to the condition mentioned earlier, the standard gamma-thujaplicin (GT-S) in chloroform (chromatographic grade) and the isolated "gamma" thujaplicin (#1, TM-006) in chloroform (chromatographic grade) were tested under the same condition with GC-MS. The testing result is only 1 peak appears for GT-S and #1 (Figure 6). Their retention time is almost the same, although with hand injecting, one is 10.744 min for GT-S, and another is 10.784 min for #1 sample. With the analysis of computer spectrum data, this peak was identified as 2,4,6-Cycloheptatrien-1-one, 2-hydroxy-5-(1-methylethyl), their match quality is above 90. It is strongly indicated that the isolated compound is gamma-thujaplicin.

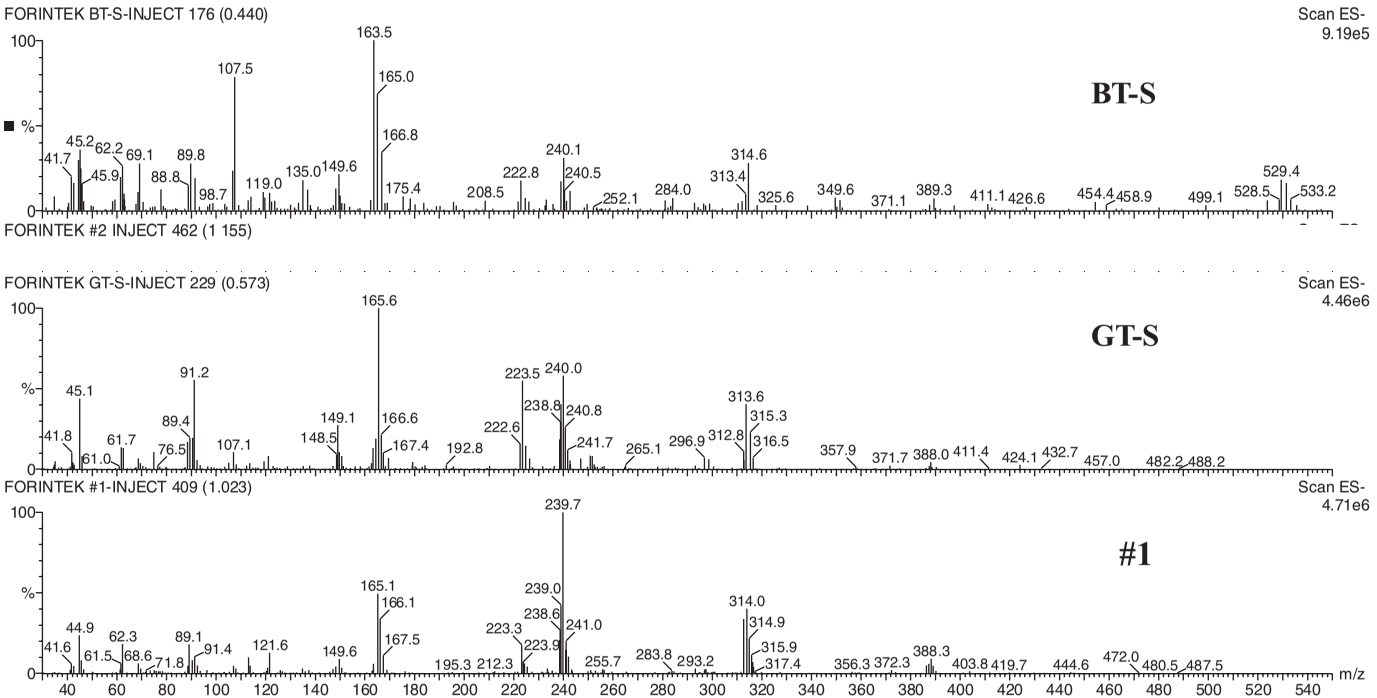


Figure 5. The MS of beta-thujaplicin standard (BT-S), gamma-thujaplicin standard (GT-S), and the isolated "gamma" thujaplicin (#1).

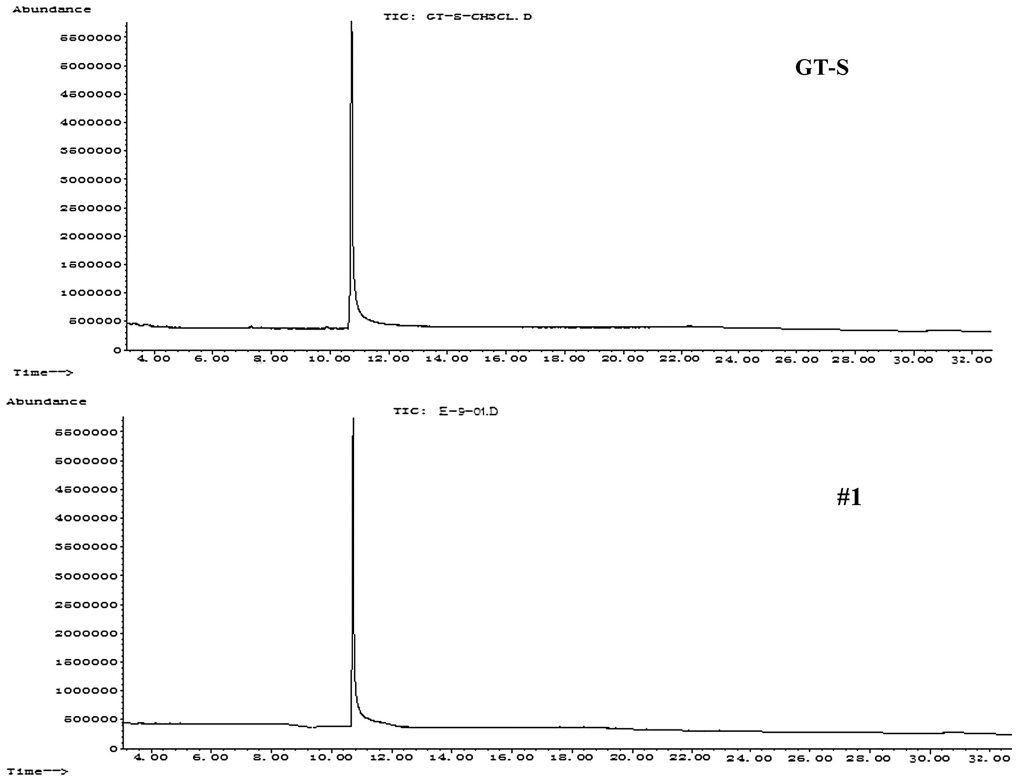


Figure 6. Total Ion Chromatogram of gamma-thujaplicin standard (GT-S) and the isolated compound (#1).

The Analysis for the Gamma-Thujaplicin with FTIR

It was observed that the peak positions and shapes were almost the same in the fingerprint zone for BT-S, GT-S, and the isolated compound in Table 1 and their IR spectrums (Figure 7). There was a minor difference at the peak intensity and position in Table 1; that is, there were three peaks for BT-S from 2961 to 2846 cm^{-1}, while GT-S has two peaks in this range, and meanwhile, the isolated compound also had two peaks in this range. From 2360 to 2108 cm^{-1}, there was

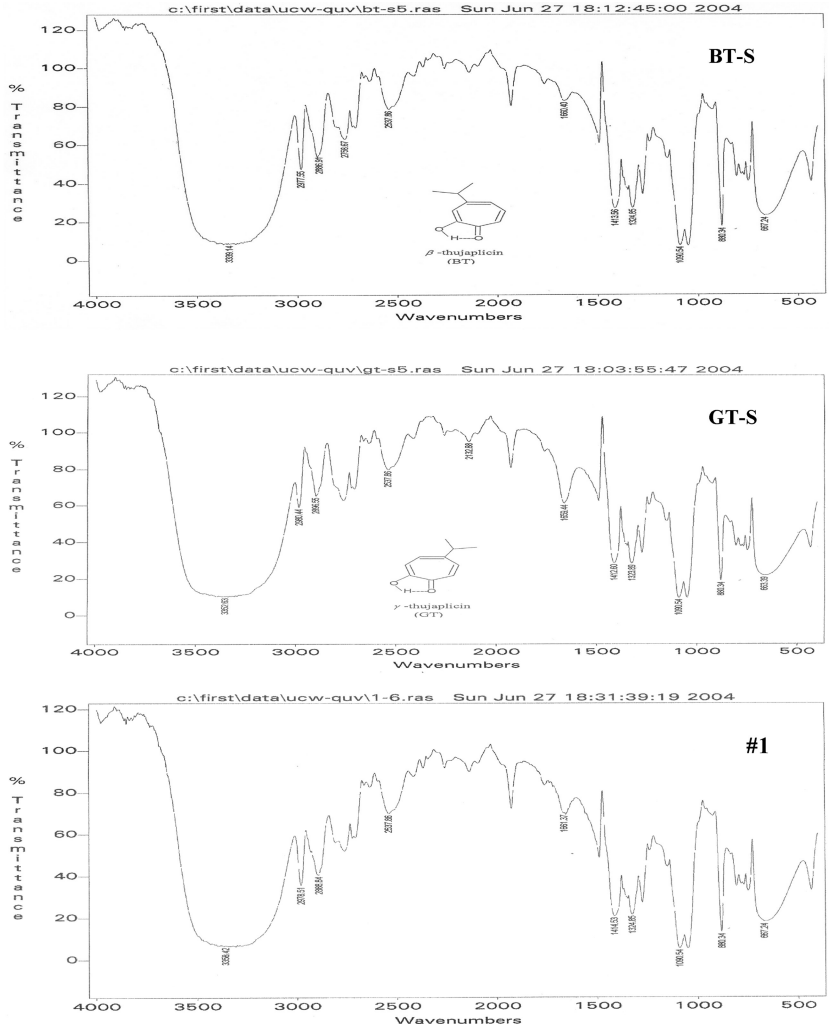


Figure 7. The FTIR of BT-S, GT-S, and the isolated compound (#1).

1 peak in the BT-S spectrum and two peaks in the GT-S and isolated compound spectra. The IR spectrums of the three samples in the fingerprint zone were identical. Although the little differences during the wavenumber ranges are not enough to prove that the isolated compound is gamma-thujaplicin, integrating the five kinds of analysis—HPLC, MS, GC-MS, 1H-NMR, and FTIR—these facts indicate that the isolated compound from WRC, sample #1, was gamma-thujaplicin.

CONCLUSIONS

The study was to isolate troplones from the heartwood of second-growth western red cedar. With n-hexane extraction and simple chemical conversion and regeneration, gamma-thujaplicin of 86–99% purity was isolated and obtained. As a by-product of the isolation of gamma-thujaplicin, nezukone was obtained in the n-hexane fraction. With modern instruments analysis, the isolated gamma-thujaplicin were characterized and identified compared with standards.

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