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Chemical analysis of extracts from Port-Orford cedar wood and bark

Heng Gao
Louisiana State University and Agricultural and Mechanical College

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CHEMICAL ANALYSIS OF EXTRACTS FROM PORT-ORFORD CEDAR WOOD
AND BARK

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The School of Renewable Natural Resources

by
Heng Gao
B.S., Qingdao University, 2001
M.S. Beijing Institute of Technology, 2004
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Abstract

This study investigated a Soxhlet extract procedure to separate chemical components in the wood and bark of Port-Orford cedar (POC) (*Chamaecyparis lawsoniana*). The bio-activities, antioxidant properties, and decay resistance were evaluated. The heartwood extracts were analyzed by gas chromatography-mass spectrometry (GC-MS). Three vitro assays including (1) 1, 1-diphenyl-2-picrylhydrazyl hydrate (DPPH•) radical scavenging assay, (2) 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation scavenging ability assay, and (3) metal chelating activity assay were carried out to evaluate the antioxidant activity. The total phenol content of the extracts was determined by the Folin-Ciocalteu method and expressed as Gallic Acid Equivalent (GAE). The results showed that the methanol extracts of the heartwood and the sapwood contained moderate radical scavenging activities, and the inner bark and outer bark extracts showed the most activities. The sapwood extracts demonstrated the strongest chelating metal capability. Among the four partitions of POC bark, the n-Butanol extracts (n-Bu) showed the highest inhibition effect on $ABTS^{+•}$ and the highest amount of total phenolic content, followed by ethyl acetate extracts (EA), water soluble components (Wa) and n-hexane extracts (n-He) subtractions. The correlation suggested that phenolic compounds are likely to contribute to the radical scavenging activity of the methanol extracts. There were three major components found in hexane extracts from the heartwood of POC, which were τ -cadinol, azulene, and τ -muurolol. Hexadecadinoic acid was found in ethyl acetate extracts. Both the n-hexane and ethyl acetate extracts of the heartwood of POC had excellent growth inhibitory effects on white- and brown-rot fungi. The n-hexane extracts showed a higher antifungal index in this assay system, which was significantly higher than the ethyl acetate extracts.

Chapter 1. Introduction

1.1. General Introduction

Secondary metabolites of trees are chemical components which are produced during complex metabolic processes. Therefore, they commonly have unique properties and very important roles in specific physical and chemical properties of wood such as color, odor, durability, and others (Wang et al. 2004). Hence, research on secondary metabolites is an important research area in wood science. This interest was increased with the discovery of the cancer fighting component, Taxol, from the bark of the Pacific yew tree. Research in secondary metabolites is largely directed to the wood science, chemistry and pharmacological fields. The research herein was based on this background. Also, another impetus for this work is the need to find an environmental-friendly wood preservative to replace traditional wood preservatives that contain heavy metals.

Port-Orford cedar (POC) (*Chamaecyparis lawsoniana*) is a large tree, 43-55 m in height and 1.2-1.8 m in diameter, restricted to the coastal forests of southwestern Oregon and northern California in the United States (Harlow et al. 1978). Botanically, it is known as *Chamaecyparis lawsoniana* and it belongs to the Cupressaceae family with other cedar and cypress species. The wood is typically straight-grained and has a characteristic pungent, “ginger-like” odor. Recent research has shown that a great number of fragrant plants contain chemical compounds, especially essential oils, exhibiting bioactivity such as antioxidant and antimicrobial properties (Dapkevicius et al. 1998, Springfield 2003, and Miliauskas et al. 2004). Therefore, to separate, isolate, and identify those effective components from the wood and bark of Port-Orford Cedar is a prelude for investigating chemical with useful bioactivities.

Antioxidants are one of the most important properties which stimulate the interests of both wood science researchers and pharmacologists. During the numerous physiological processes, some free radical and reactive oxygen species (ROS) are produced which are harmful for some biomolecules and can cause cardiovascular diseases (Frei 1995). An antioxidant is the hydrogen donating ability which can reduce free radicals and make them stable. After the free radicals are reduced by antioxidants, they are no longer harmful to human health. Therefore antioxidants are effective to

prevent many diseases and are becoming an increasingly important in the pharmacological chemistry area. Chapter 2 presents the antioxidant activities of extracts of wood and bark of POC. In order to get antioxidant results in broad range, we try three in-vitro assays (including (1) 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH•) radical scavenging assay, (2) 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation scavenging ability assay and, (3) metal chelating activity to test the activities. Both the DPPH• and ABTS⁺ are stable free radicals and are widely used to evaluate radical-scavenging activities. The methods are based on the chemical reactions of a solution of free radical (DPPH• or ABTS⁺ in this research) with a hydrogen donating antioxidant to reduce those free radical into non-radical form. This transformation results in a change of color which is measured spectrophotometrically at a specific wavelength. Because metal chelating activity is another kind of antioxidant activity, it was also determined by a ferrozine-Fe²⁺ reaction system.

Another important property of secondary metabolites is antifungal activity. Wood products are degraded by many organisms which causes economic losses every year. The traditional metal-based wood preservatives have been removed from general public use. Therefore, research on chemical components and their antifungal activities of inherent decay resistance tree species is becoming an important field in wood preservation. Chapter 4 presents the evaluation of the antifungi activities including white- and brown-rot fungi decay resistance. Chapter 3 also presents the components analysis by using gas chromatography-mass spectrometry (GC-MS).

1.2. Objectives

The overall objective of this study was to conduct extract and chemical analysis and evaluate antioxidant and antifungal activities of the wood and bark of POC. The extract procedures, extract yields, and bioactivities were investigated. The study consisted of the following specific objectives.

1. Extract the antioxidant components from heartwood, sapwood, inner bark, and outer bark of POC and evaluate the antioxidant activities of those extracts. The evaluation method included DPPH• radical scavenging assay, ABTS radical cation scavenging ability assay, and metal chelating activity assay.

2. Perform advanced isolation work on bark crude extract due to its higher antioxidant activity. Four different solvents, (n-hexane, ethal acetate and n-BuOH) were used to obtain subtractions. The (DPPH•) radical scavenging and (ABTS) radical cation scavenging ability were tested in order to evaluate antioxidant activity.
3. Separate heartwood of POC by sequentially extracting with hexane and ethyl acetate at the temperature of 68 °C and 77 °C and identify the chemical components in the extracts by GC-MS.
4. Evaluate the antifungal activity (decay resistance) of the heartwood sequential extracts. White- and brown-rot fungi were used. The antifungal index (AI) was tested to indicate the performance of the antifungal activity.

1.3. References

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Chapter 2. Antioxidant Analysis of Extracts of Wood and Bark of POC

2.1. Introduction

Free radicals and reactive oxygen species (ROS) are byproducts of numerous physiological and biochemical processes. Some reports have shown that excessive ROS may be harmful for some biomolecules and cause or promote aging (Aruoma 1994), cancer (Pietta 2000), and cardiovascular diseases (Frei 1995). Also, ROS can cause lipid peroxidation in foods, which leads to deterioration in food quality, affecting the color, flavor, texture and nutritive value of the food (Sasaki et al. 1996). In order to reduce damage of ROS to the human body and prolong the storage stability of foods, antioxidants are used for industrial food processing (Yu et al. 2002). Synthetic antioxidants such as 2, 3-tert-butyl-4-methoxy phenol (BHA) and 2, 6-di-tert-butyl-4-methyl phenol (BHT) are widely used in the food industry. However, previous research has reported a toxic effect of some commonly used synthetic antioxidants, which show carcinogenic effects in living organisms (Grice 1986, Wichi 1998, Williams et al. 1999). Hence, there is a great need to search for safe and natural antioxidants for the food, cosmetic, and medicinal industries.

Polyphenol compounds are reported to be a good source for natural antioxidants (Hagerman et al. 1998). Some researchers have investigated the relationship between antioxidant activity and polyphenol content. Natural antioxidants have been found in a number of food and agricultural products including tea, spices, oilseeds, and vegetable and cereal grains. (Cao et al. 1996, Wang et al. 1996). Besides these traditional resources used for antioxidants, many plant species have been investigated in the search for natural antioxidants (Baniyas et al. 1992). The discovery of Taxol from bark of the Pacific yew tree stimulated the interest in antioxidants from woody plants and other medicinal plants for cancer fighting applications for humans.

In this chapter, wood and bark of Port-Orford cedar (POC) (heartwood, sapwood, inner bark, and outer bark) were extracted with methanol and the antioxidant activities were evaluated based on different free radicals. Sequential separation using n-hexane, ethyl acetate, and n-BuOH of the bark extracts was conducted due to its higher activities.

Also total phenolic content of methanol extracts was determined according to the Folin-Ciocalteu method.

2.2. Materials and Methods

2.2.1 Chemicals

Potassium peroxydisulfate, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4-disulfonic acid, and sodium salt (ferrozine) were purchased from Sigma Chemical Co. (St. Louis, Mo USA), ferrous chloride, gallic acid, 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH•), and Ethylenediaminetetraacetic acid dihydrate, disodium salt were purchased from Alfa Aesar (Ward Hill, MA, USA), and butylated hydroxytoluene (BHT) was purchased from Alfa Aesar (A Johnson Matthey Co., Lancaster, England). All of the other chemicals were of standard analytical grade except the methanol used in the DPPH• assay was HPLC grade and the ethanol used in the ABTS test was spectrophotometry grade.

2.2.2 Preparation of Crude Extract of Wood and Bark

POC wood was collected from a 10 cm-thick disk of a log and stored at -4 °C. The outer bark of the strips was first removed with a fine blade. The rest of the strips were divided using a fine blade based on outer bark, inner bark, sapwood and heartwood along the radial direction. The samples were initially air-dried and then cut into small strips with a razor blade. All of these materials were ground in a Wiley Mill. The material was then placed in a shaker with sieves. The material that passed through a No. 40 mesh sieve (425-µm) yet retained on a No. 60 mesh sieve (250-µm) was collected. The resulting material was placed in glass jars and labeled. Soxhlet extraction of particles was conducted with methanol until the solvent became colorless. Then, all of the extracts were collected, dried under a rotary evaporator, lyophilized in air freeze drier, and kept in the dark at 4°C until testing. The following formula was used to determine the extract yield.

$$\text{Extract yield (\%)} = \frac{W_1}{W_2} \times 100 \quad [1]$$

Where

W_1 = net weight of extracts (grams)

W_2 = total weight of wood powder (grams)

2.2.3 Preparation of Advanced Separation of Bark Extracts

The bark crude extracts were concentrated, suspended in water, and sequentially partitioned with n-hexane, ethal acetate, and n-BuOH to obtain four different polar subtractions, denoted by n-He, EA, n-Bu, respectively, and Wa (water soluble components). Subtractions were collected, dried under a rotary evaporator, lyophilized in an air freeze drier, and kept in the dark at 4°C until testing. The following formula was used to determine the extract yield.

$$\text{Extract yield (\%)} = \frac{W_3}{W_4} \times 100 \quad [2]$$

Where

W_3 = net weight of subtractions (grams)

W_4 = total weight of bark sample (grams)



Figure 2-1. Picture of the rotary evaporator.

2.2.4 Evaluation of Antioxidant Activities

DPPH• radical scavenging assay

This method is based on the reduction of a methanol solution of DPPH• (1, 1-diphenyl-2-picrylhydrazyl hydrate) in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH-H (Koleva et al. 2002, Soler-Rivas et al. 2000). This transformation results in a change of color from purple to yellow, which is measured spectrophotometrically by the disappearance of the purple color at 517 nm. Methanol solutions (0.1 mL) of the extracts at various concentrations were added to 5 mL of a methanol solution of DPPH• free radical or methanol alone (blank) (Burits and Bucar 2000, Cuendet et al. 1997). The reaction mixture was vigorously shaken by hand and then kept in the dark for 30 min. at ambient conditions. The absorbance was measured at 512 nm, and the antioxidant capacity was expressed as inhibition %, which was calculated by the following formula.

$$\%Inhibition = \left(\frac{A_0 - A_1}{A_0} \right) \times 100 \quad [3]$$

Where

A₀= absorbance without extracts

A₁= absorbance with extracts at 512 nm

The IC₅₀ is the antioxidant concentration that inhibits the DPPH• reaction by 50% under the experimental conditions. This was calculated by plotting the inhibition percentage against extract concentration. Low IC₅₀ values indicate high free radical scavenging activities. In this experiment, a synthetic antioxidant reagent, butylated hydroxytoluene (BHT), was used as a positive control. All analyses were run in three replicates and averaged.

ABTS⁺ radical cation scavenging

The ABTS⁺ radical scavenging test is widely used to determine the antioxidant activity of both hydrophilic and lipophilic compounds. The reaction between ABTS⁺ and potassium persulfate directly generated the blue/green ABTS⁺ chromophore, which can be reduced by an antioxidant thereby resulting in a loss of absorbance at 734 nm. The experiment was carried out according to an improved method described by Re et al. (1999) with some modification. ABTS⁺ was generated by mixing 5 mL of 7 mM ABTS

with 88 μL of 140 mM $\text{K}_2\text{S}_2\text{O}_8$ under darkness at room temperature (23°C) for 16 h. The solution was diluted with 50% ethanol to an absorbance of 0.7 ± 0.05 at 734 nm. The $\text{ABTS}^{\cdot+}$ radical cation scavenging activity was assessed by mixing 5 mL $\text{ABTS}^{\cdot+}$ solution (absorbance of 0.7 ± 0.05) with 0.1 mL bark extracts or negative control (methanol). The final absorbance was measured at 734 nm. The inhibition percentage of $\text{ABTS}^{\cdot+}$ was calculated by Equation 1 with slight modifications. A_0 equals the absorbance without extracts at 734 nm and A_1 equals the absorbance with extract or BHT at 734. All experiments were performed in triplicate.

Analysis of metal chelating activity

The chelating activity of extracts was estimated according to Dinis et al. (1994). The extracts with different concentration in methanol were incubated with 0.05 mL FeCl_2 (2.0 mM). The reaction mixture was initiated by the addition of 0.2 mL of ferrozine (5 mM) and kept at room temperature. The absorbance was then read at 562 nm after the reaction mixture reached equilibrium in 10 min. Ethylenediaminetetraacetic acid dihydrate, disodium salt ($\text{EDTA}\cdot\text{Na}_2\cdot 2\text{H}_2\text{O}$) served as a positive control, and a sample without extract and $\text{EDTA}\cdot\text{Na}_2\cdot 2\text{H}_2\text{O}$ served as negative controls. Triplicate samples were run for each set and averaged. The percentage inhibition of ferrozine- Fe^{2+} complex formation was calculated based on Equation 1 in which A_0 equals absorbance without extracts, and A_1 equals absorbance with extracts at 562 nm.

Determination of Total Phenolic Content (TPC)

The total phenolic content of methanol crude extracts was determined according to the Folin-Ciocalteu method described by Singh et al. (2002) with slight modification. The result was expressed as gallic acid equivalents (GAE), which reflect the phenolic content as the amount of gallic acid (mg/g) dry weight of sample. Methanol solution of extracts (0.5 mL) was mixed with tenfold diluted Folin-Ciocalteu reagents (2.5 mL) and incubated for 2 minutes at room temperature before the addition of a sodium carbonate solution (2 mL, 7.5% w/v). The absorbance of the mixture solution was measured at 765 nm after standing for 30 minutes at room temperature. Gallic acid solutions (0.5 mL) with a concentration range of 0.2-0.025 $\text{mg}\cdot\text{mL}^{-1}$ were used to make the calibration curve. The experiment design was the same as extracts. The experiment was carried out in triplicate, and the results were averaged.

2.3. Results and Discussion

Table 2-1 and 2-2 show the extract yield of crude extract (heartwood, sapwood, inner bark, and outer bark) and advanced separation of bark subtractions (n-He, EA, n-Bu, and Wa). The results of DPPH• scavenging ability, ABTS⁺• scavenging ability, and metal chelating activity of crude extracts of wood and bark of POC are listed in Figure 2-2, 2-4, and 2-6. According to the antioxidant results of crude extracts, the outer bark did not show good metal chelating activity. In the next step test of antioxidant activity for bark subtractions, only DPPH• scavenging and ABTS⁺• scavenging assay were tested. The results are shown in Figure 2-3 and 2-6. Figure 2-7 and 2-8 show the correlations of total phenol content and IC₅₀ values of wood and bark extracts.

2.3.1 Soxhlet Extract Yields

The crude extracts of heartwood, sapwood, inner bark, and outer bark of POC had different colors. The heartwood extracts showed yellow oil character; sapwood extracts were a light yellow powder and had the lowest yield (1.0%). Therefore, more raw sapwood materials (113.0742 g) were used to conduct the extract step in order to get enough products to do the next antioxidant activity evaluation. Inner bark extracts showed red color with the highest yield (16.5%) and the outer bark extract were a dark red powder also with a high yield (15.9%).

The advanced separation yields of n-He, EA, n-Bu, and Wa of bark were 5.077, 7.739, 2.421 and 0.6423 (w/w), respectively. N-Hexane extracts were a light green oil and viscous. Ethyl acetate was the most effective solvent to get the most of the subtraction. Because n-BuOH has a very high boiling point (140°C), it is difficult to remove n-BuOH from the mix solution. In this research, deionized water was added to n-BuOH solution (63% n-BuOH+37% water) to make n-BuOH and H₂O boil at a lower temperature and protect n-Bu from degradation at the high temperatures.

Table 2-1 Soxhlet extract yield of wood and bark of POC.

Items	Heartwood	Sapwood	Inner bark	Outer bark
Net weight of wood powder (g)	48.2174	113.0742	28.5339	36.0261
Net weight of extracts (g)	1.8853	1.1229	4.7117	5.7192

Extract yield (%)	3.9	1.0	16.5	15.9
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Table 2-2. Advanced separation yield from bark subtractions.

Items	n-He	EA	n-Bu	Wa
Net weight of bark powder (g)	89.8700	89.8700	89.8700	89.8700
Net weight of subtractions (g)	4.5631	6.9550	2.1760	0.5772
Extract yield (%)	5.077	7.739	2.421	0.642

2.3.2 Effect of DPPH• Radical Scavenging Activity

DPPH• is a stable free radical and is widely used to assess the radical-scavenging activity of antioxidant compounds. This method is based on the reduction of a methanol solution of DPPH• in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH-H (Koleva et al. 2002, Soler-Rivas et al. 2000). This transformation results in a color change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple color occurs at 517 nm.

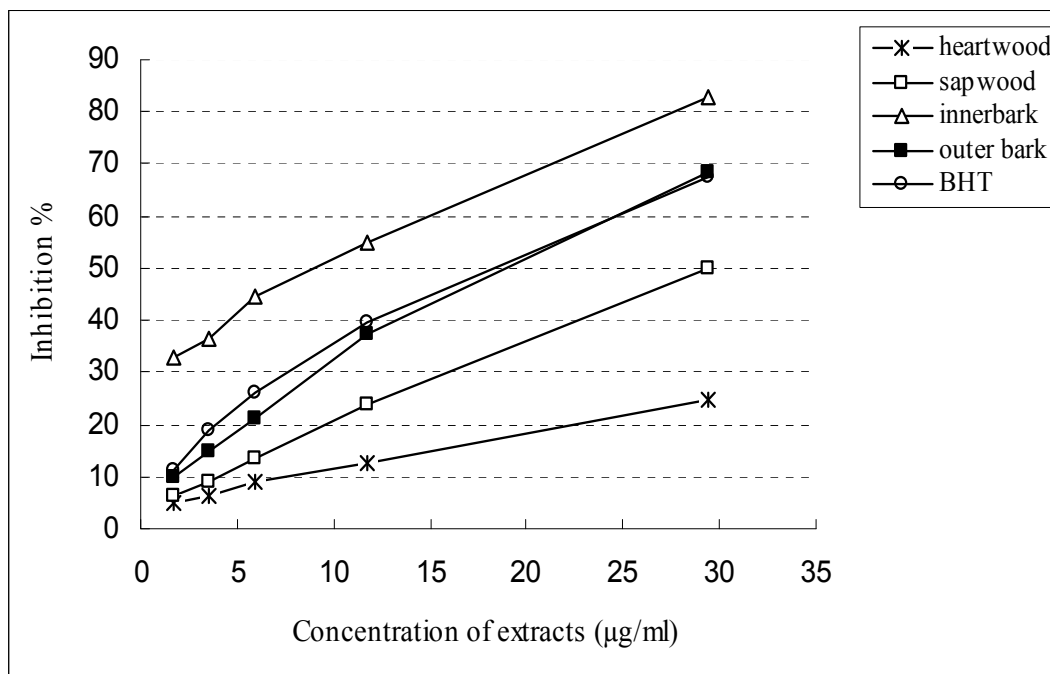


Figure 2-2. DPPH• scavenging capability of different extracts of POC.

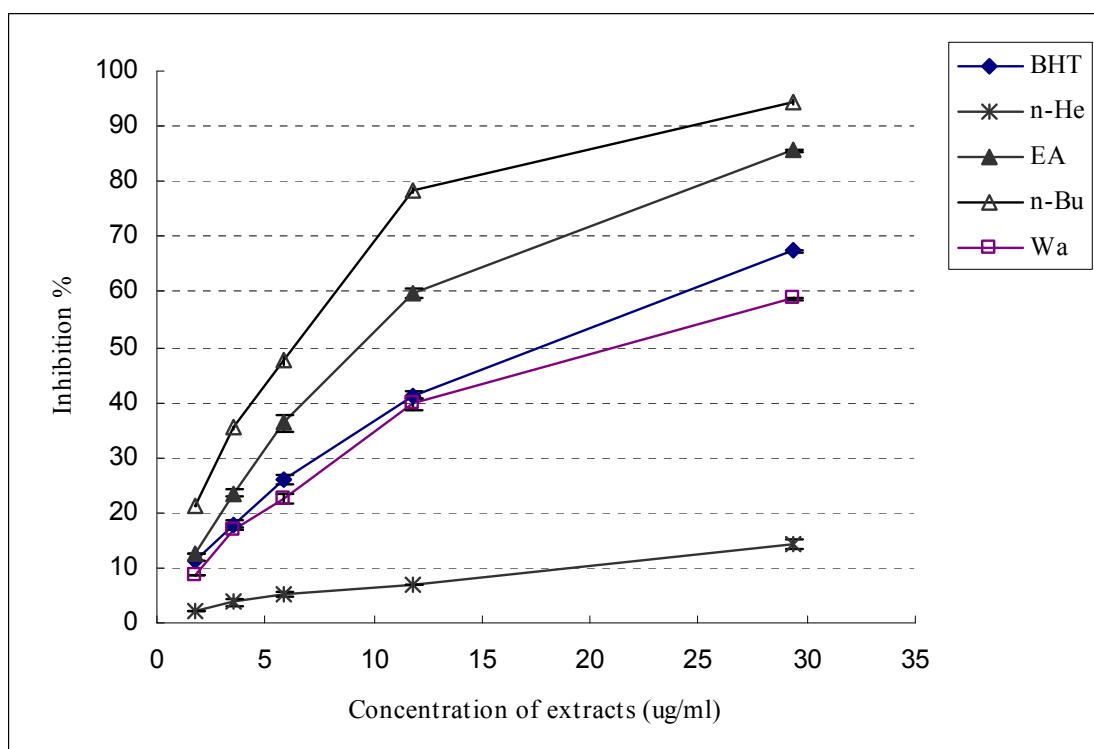


Figure 2-3. DPPH• scavenging capability of the bark substractions.

In Figure 2-2, all POC extracts exhibited concentration-dependent DPPH• radical scavenging activity. Among the extracts isolated, the heartwood and the sapwood revealed moderate antioxidant activities in this assay system with inhibition percentages of 24.75 and 50.05 %, respectively. The inner bark extract showed the most activity (82.62%), which was significantly higher than the BHT positive control (67.45%). The results showed that the radical scavenging activity of the outer bark extract was close to that of BHT. The IC₅₀ values of heartwood, sapwood, inner bark, and outer bark extract were 64.77 $\mu\text{g}\cdot\text{mL}^{-1}$, 29.03 $\mu\text{g}\cdot\text{mL}^{-1}$, 10.31 $\mu\text{g}\cdot\text{mL}^{-1}$, and 19.87 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. The IC₅₀ value of heartwood was obtained by extrapolation of the data because of its low antioxidant activity.

According to Figure 2-3, the EA and n-Bu substractions showed high antioxidant activities with inhibition percentages of 85.61 and 94.52 %, respectively, at a concentration of 29.41 $\mu\text{g}\cdot\text{mL}^{-1}$. Wa revealed moderate activity, which is close to that of BHT, while the n- He subtraction showed weaker activity compared to the other three substractions and the positive control. The IC₅₀ values of n-He, EA, n-Bu, and Wa

subtractions were $115.4 \mu\text{g}\cdot\text{mL}^{-1}$, $13.04 \mu\text{g}\cdot\text{mL}^{-1}$, $6.53 \mu\text{g}\cdot\text{mL}^{-1}$, and $22.48 \mu\text{g}\cdot\text{mL}^{-1}$, respectively. The IC_{50} value of the reference compound, BHT, was approximately $19.27 \mu\text{g}\cdot\text{mL}^{-1}$. The n-Bu subtraction showed the lowest IC_{50} , which indicated it was the most effective against DPPH• radical compared with the well-known antioxidant BHT. The IC_{50} value of n-He was obtained by extrapolation of the data because of its low antioxidant activity.

2.3.3 Effect of $\text{ABTS}^{\cdot+}$ Radical Ration Scavenging Activity

$\text{ABTS}^{\cdot+}$ radical cation scavenging assay is a rapid and reliable method that is widely used in the total radical-scavenging measurement of pure substances, aqueous mixtures, and beverages. The reaction between $\text{ABTS}^{\cdot+}$ and potassium persulfate directly generated the blue/green $\text{ABTS}^{\cdot+}$ chromophore which can be reduced by an antioxidant, thereby resulting in a loss of absorbance at 734 nm. The Re et al. (1999) method was applied in this study to obtain the radical-scavenging data shown in Figure 3-b and 5-b.

According to Figure 2-4, the control, BHT, and the four extracts exhibited concentration-dependent $\text{ABTS}^{\cdot+}$ radical-scavenging activity. At concentrations from $1.765 \mu\text{g}\cdot\text{mL}^{-1}$ to $5.882 \mu\text{g}\cdot\text{mL}^{-1}$, the slopes of the curves of inhibition percentage versus concentration of inner bark and BHT were larger than those of the other three extracts and demonstrated that in that concentration range the anti-radical activity increased rapidly with greater sample concentration. The slope increased slowly or became constant at higher concentrations, from 5.882 to $29.41 \mu\text{g}\cdot\text{mL}^{-1}$. In these instances, the $\text{ABTS}^{\cdot+}$ may have been mostly reduced and the color was not proportional to the amount of radical scavenger. Among the four extracts of POC, the inner bark showed an inhibition effect on $\text{ABTS}^{\cdot+}$ at all test concentrations that were higher than the synthetic antioxidant, BHT, in the same experimental condition, followed by outer bark and sapwood extracts. The heartwood extract showed the weakest radical-scavenging ability.

The $\text{ABTS}^{\cdot+}$ scavenging abilities of bark advanced separation components are shown in Figure 2-5. The overall changing trends of $\text{ABTS}^{\cdot+}$ scavenging activity of bark advanced separation is the same as that of crude extracts. Among the four partitions of POC bark, n-Bu showed the highest inhibition effect on $\text{ABTS}^{\cdot+}$ followed by EA, Wa, and n-He subtractions.

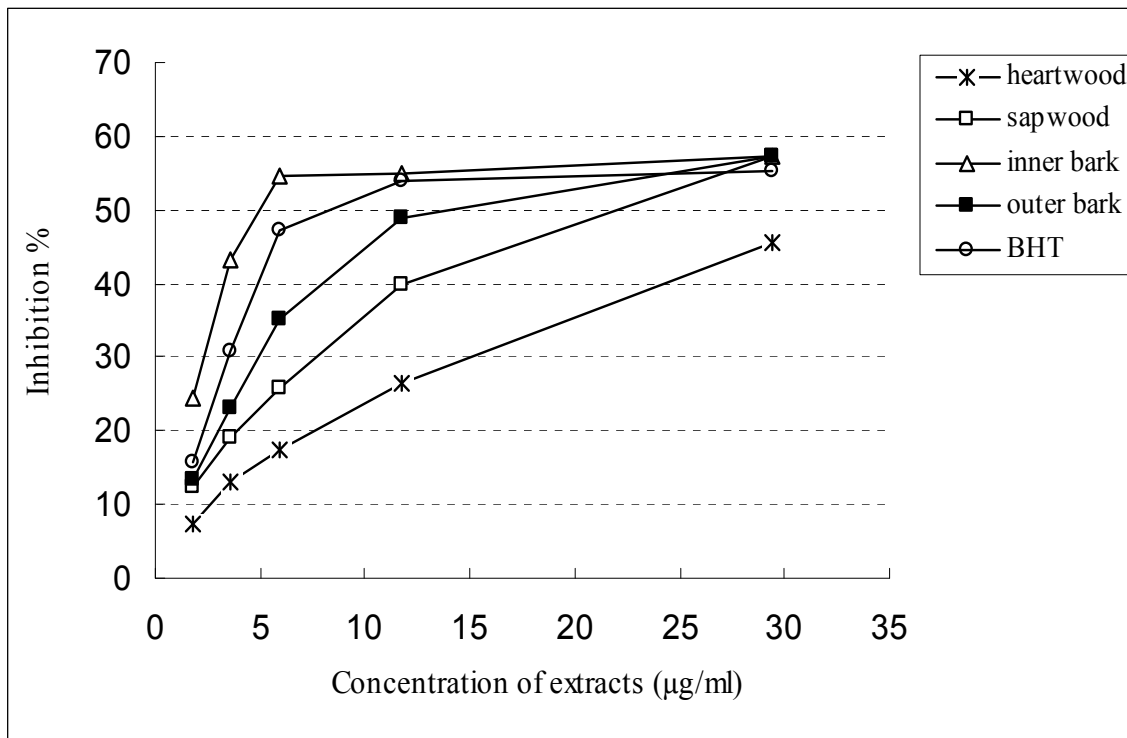


Figure 2-4. ABTS⁺ scavenging capability of different extracts of POC.

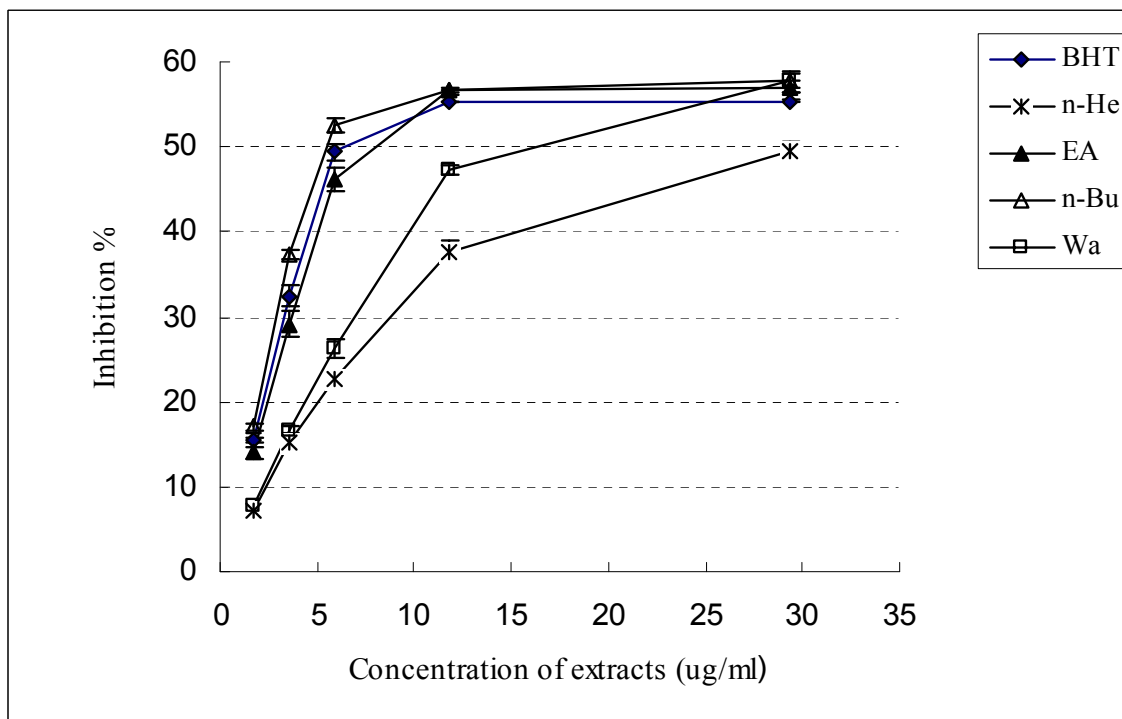


Figure 2-5. ABTS⁺ scavenging capability of the bark subtractions.

2.3.4 Metal Chelating Activity

Ferrozine can quantitatively chelate with Fe^{2+} and form complexes with a red color. This reaction is limited in the presence of other chelating agents and results in a decrease in the intensity of the red color of the ferrozine- Fe^{2+} complexes. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions (Yanmaguchi et al. 2000). The chelating abilities of samples and EDTS- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$ are given in Figure 2-6. It should be noted that of the four samples, sapwood extract demonstrated the strongest chelating metal capability. The heartwood, outer bark, and inner bark showed negligible metal chelating activity.

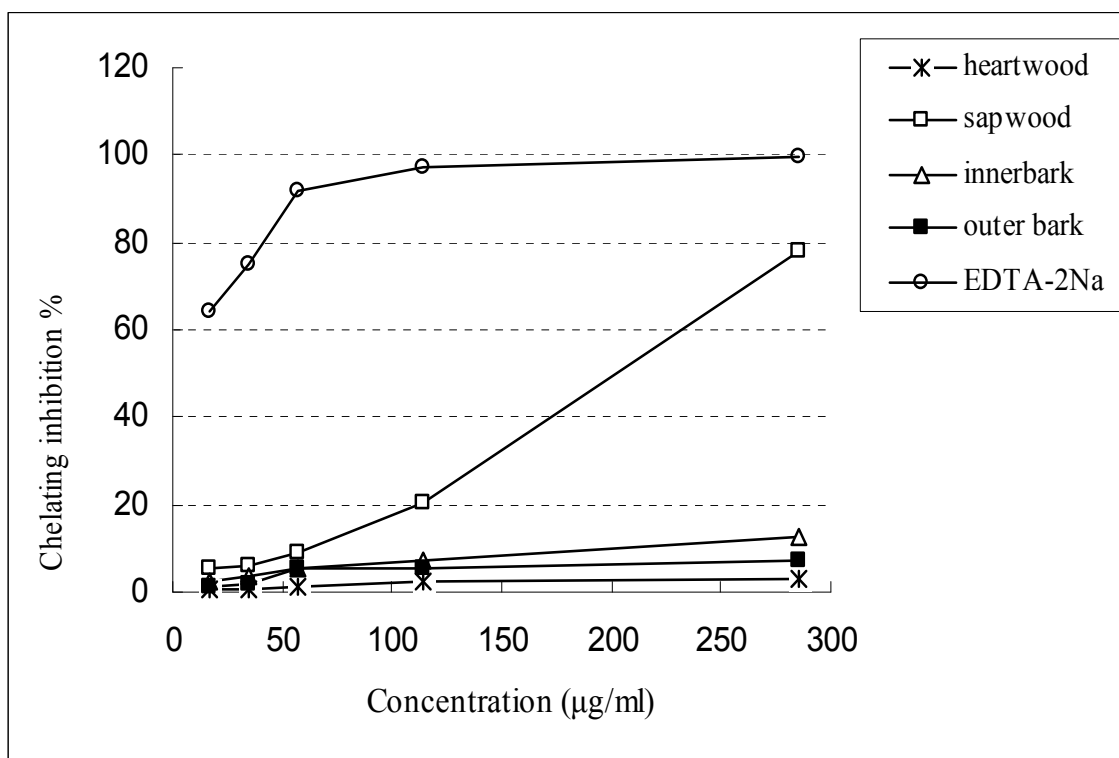


Figure 2-6. Chelating metal capability of different extracts of POC.

Transition metal ions may act as catalysts that promote the generation of radicals thereby initiating oxidative chain reactions. The chelating agents, which form σ -bonds with metals, can stabilize the oxidized form of the metal iron and then decrease the possibility of oxidative chain reactions (Gordon 1990). The present study detected significant chelating activity against Fe^{2+} in the sapwood extract. This may be because sapwood is a living tissue part in the tree stem and has the function of transferring water

and other materials during the course of photosynthesis. Therefore, it is reasonable that there are some special secondary metabolites in the sapwood which have high chelating capability. This may infer high potential development for biological or food systems in order to safeguard human health and food quality and safety.

2.3.5 Total Phenol Content of POC Extracts

The content of phenolics ($\text{mg}\cdot\text{g}^{-1}$) in methanol extracts was determined from a regression equation of the calibration curve ($y = 0.004663x + 0.0565$, $R^2 = 0.99$) and expressed in Gallic Acid Equivalents (GAE). Table 2-3 and 2-4 show that the distribution of phenolic compounds in the four extracts from POC and in the four subtractions. Figure 2-7 and 2-8 show the relationship between the phenolic content and the IC_{50} of DPPH• radical scavenging assay. (Figure 2-7 shows the crude extracts and Figure 2-8 presents the data for the subtractions).

Table 2-3. TPC of different extracts from wood and bark of POC.

Sample	mg GAE $\cdot\text{g}^{-1}$ dry extract	mg GAE $\cdot\text{g}^{-1}$ dry wood powder
Heartwood	136.9	5.353
Sapwood	257.7	2.559
Inner bark	537.5	88.76
Bark	489.1	77.65

Table 2-4. TPC of different extracts from bark subtractions.

Sample	mg GAE $\cdot\text{g}^{-1}$ dry extract	mg GAE $\cdot\text{g}^{-1}$ dry bark powder
n-He	65.7	3.336
EA	337.0	26.08
n-Bu	428.5	10.38
Wa	193.8	1.245

Note: TPC, Total phenol content; GAE, Gallic Acid Equivalents. n-He, EA, n-Bu, and Wa are the abbreviation for the subtractions of the n-hexane, ethal acetate, n-BuOH, and water respectively.

The inner bark contained the highest amount of total phenol content, 537.5 mg GAE per g of extract, followed by outer bark, sapwood and heartwood (Table 2-3). However, the GAE per g dry wood powder of the sapwood showed the lowest value because it had the lowest extract yield (0.9931%, w/w) among the four samples. The correlation suggests that phenolic compounds are likely to contribute to the radical scavenging activity of the methanol extracts. As can be seen in Table 2-4, the distribution of phenolic compounds in the four extractions from bark of POC demonstrated that n-Bu contained the highest amount, 428.5.5 mg GAE per g of extract, followed by EA, Wa, and n-Bu. However, the GAE per g dry bark powder of EA showed the highest value because it had the highest extract yield (7.739%, w/w) among the four samples. Therefore, ethyle acetate is a good solvent for separating antioxidant polyphenols.

Figure 2-7 shows the relationship between the phenolic content in the extracts and the IC₅₀ of DPPH• radical scavenging assay. The correlation showed that the IC₅₀ value of DPPH• radical scavenging has a linear relationship with the polyphenol compounds content ($y = -0.1149x + 71.805$ $R^2 = 0.84$).

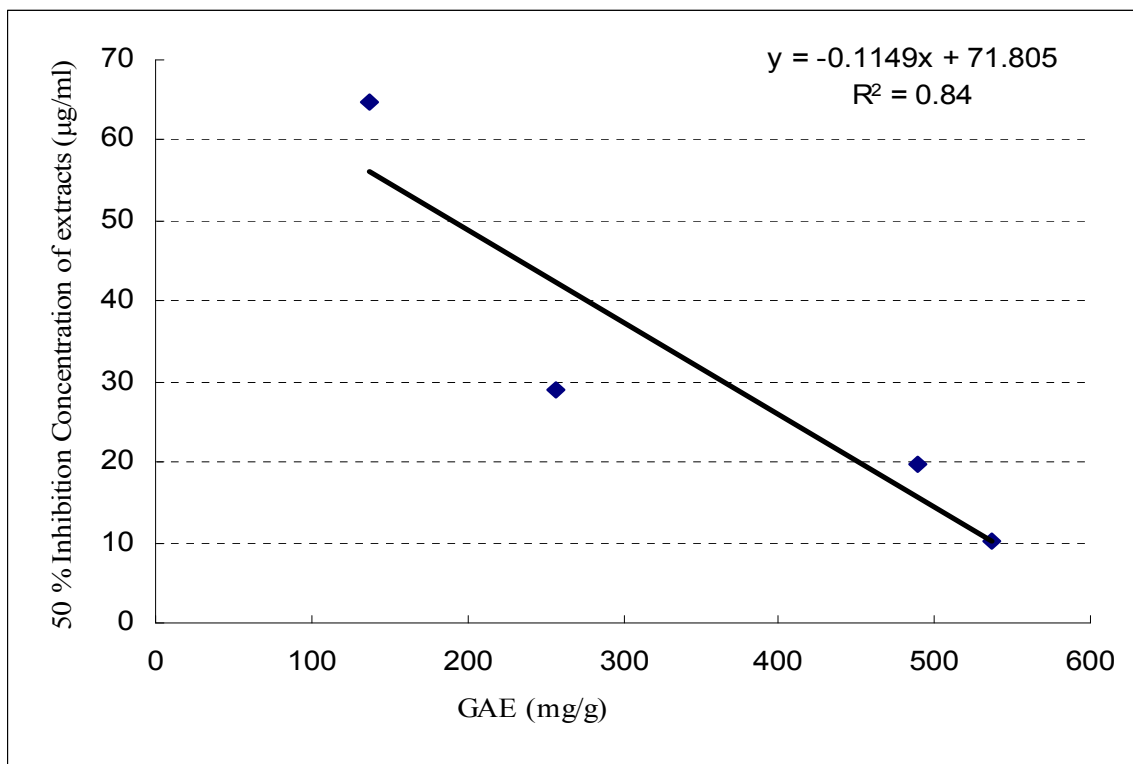


Figure 2-7. Correlation of TPC and IC₅₀ values (DPPH•) of extract.

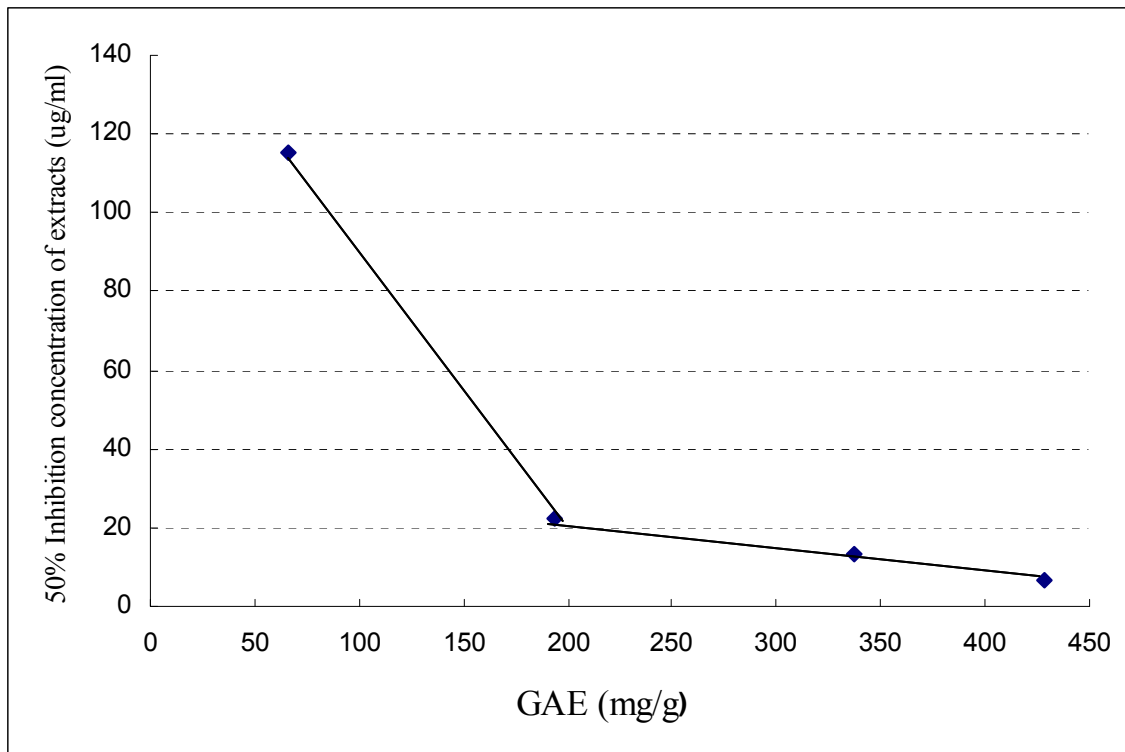


Figure 2-8. Correlation of TPC and IC₅₀ values (DPPH•) of extractions

The correlation showed that the IC₅₀ value of DPPH• radical scavenging decreases with an increase in the polyphenol compounds content. The correlation suggests that phenolic compounds are likely to contribute to the radical scavenging activity of the methanol extracts.

2.4. Summary

Extracts from wood and bark are complex natural products. Generally, inner bark and sapwood are rich in nutrients such as sucrose and glycosides. Heartwood and outer bark, by contrast, tend to be deficient in nutrients, but are rich in compounds such as hydrolysable and condensed tannins and many other phenolics capable of protecting the tissues against biological attack. In the broadest sense, all secondary metabolites are taxonomically significant.

The results showed that methanol extracts exhibited promising radical scavenging activity. More specifically, the inner bark demonstrated the strongest antioxidant activity and the greatest total phenolic content among the four extracts and the positive control.

The antioxidant mechanisms of POC extracts may be due to the strong hydrogen donating ability of the phenol compound contained in the POC and can reduce the *DPPH*• and *ABTS*⁺• free radicals.

Different polar extractions n-Hex, EA, n-Bu and Wa of POC bark were examined by *DPPH*• and *ABTS*⁺• methods for their free radical scavenging activity. The results showed that ethyl acetate has the highest extraction yield among the other two solvents and the second was n-BuOH. The n-Bu and EA extraction demonstrates the strong antioxidant activity. Compared with wood or leaves, bark is the most economical and convenient resource to find antioxidant compounds. Approximately 18 percent the weight of a conifer wood is bark (Hemingway 1997). However, the primary use of bark is to burn as fuel in spite of its low fuel value (Karchchesy and Koch 1979). According to this research, the bark extractions have good antioxidant activities and hence bark has potential as a natural source of antioxidants.

2.5. References

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Chapter 3. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Heartwood Extracts of POC

3.1. Introduction

Recent research has shown that a great number of fragrant plants contain chemical compounds, especially essential oils, exhibiting bioactivity such as antioxidant and antimicrobial properties (Dapkevicius et al. 1998, Springfield 2003, Miliauskas et al. 2004). According to the findings of Chapter 2, heartwood extracts of POC were oily with a light yellow color and fragrant odor. Combining previous research and the special characters of the wood oil, I believe that the chemicals in the heartwood of POC are important for its natural decay resistance. Therefore, it is necessary to do advanced extract work and chemical analysis on the heartwood oil.

There are two possible methods to conduct the extraction work on oil components in plants. One is steam-distillation (Figure 3-1) and the other is a Soxhlet extractor (Figure 3-2). In a steam-distillation system, the boiling steam passes through the samples and removes the volatile compounds in the wood powder. The hot gas mixture travels through the condensing coil and is converted back into a liquid, which is collected in the receiver flask. Then the crude extract is dried by using anhydrous sodium sulphate. Though steam-distillation can work well on the extracts of volatile compounds, such as essential oils and other aromatics, it can not give a good extract yield for compounds with higher boiling points.

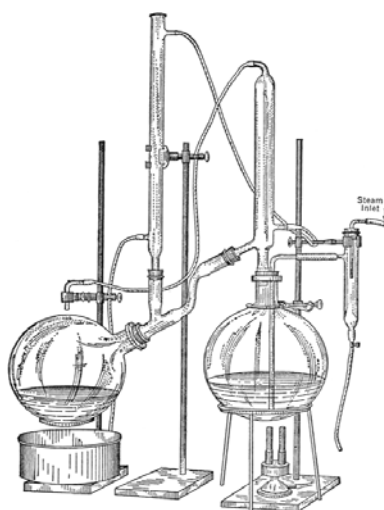


Figure 3-1. Schematic of steam-distillation.

Compared with the steam distillation method, Soxhlet extraction has key advantages because the later can increase the extraction efficiency and has the capability to extract different compounds if an appropriate solvent is selected. In the Soxhlet process, an extractor chamber was connected to a condenser on the top and a flat bottom flask at the bottom, the later of which contains the solvent. An extractor thimble (33×80mm) was filled with the test sample and placed inside of the extraction chamber. The solvent was heated to evaporate. The hot solvent vapor moved up to the condenser, where it was cooled and dripped into the sample particles which were slowly filled with warm solvent. When the liquid height reached the siphon height, siphon action occurred and the liquid flew back down into the flask. This cycle was repeated many times. During each cycle, a portion of the compounds in the sample particles was dissolved in the solvent. However, once the extraction mixture reached the solvent heating flask, the extracted compounds remain in the flask and would not participate in solvent cycle any further. In this research, because the effective components for decay resistance of POC are unknown, the best way to find them was to extract all components with different polarity solvents and then evaluate them one by one. In this project, the solvents hexane and ethyl acetate were used as the extract solvents in increasing polarity to obtain extracts with a broad range. The lower polarity solvent, hexane, was used for the extractin in the first step, and the higher polarity ethyl acetate was used to do the second extraction step.

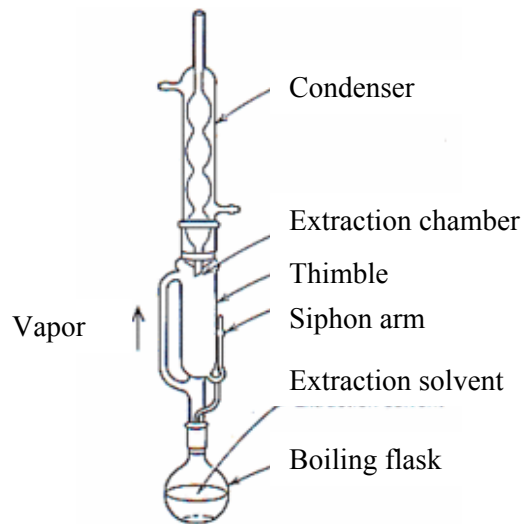


Figure 3-2. Schematic of Soxhlet extraction.

3.2. Materials and Methods

3.2.1 Chemicals

Hexane and ethyl acetate used for the extract work were standard analytical grade. The solvent chloroform was used for dissolving the extract oil to conduct GC-MS. The GC-MS used for this research to detect the extracts was a Varian GC/MS system.

3.2.2 Sample Preparation

The heartwood of POC was collected from a 10 cm-thick disk of a log and stored at -4 °C. The samples were initially air-dried and then cut into small strips with razor blade. All of these materials were ground in the Wiley Mill. The material was then placed in a shaker with sieves to pass through a No. 40 mesh sieve (425-µm) yet be retained on a No. 60 mesh sieve (250-µm). The resulting material was placed in glass jars and labeled with appropriate codes for Soxhlet extraction.

3.2.3 Soxhlet Extraction Using Hexane

Soxlet extraction of wood particles at was conducted 68 °C with hexane until the solvent was colorless. Boiling stones was added to the flat bottom flasks to prevent sudden boiling. The extraction work was done on a six station heating bank. The extraction mixtures were collected and the thimble was rinsed three times using hexane to make sure all of the extract solution went into the collection containers. The solid residues after the hexane extractions were air dried prior to ethyl acetate extraction. The following formula was used to determine the extraction yield using hexane. Table 3-1 shows the weight of wood materials for hexane extracts.

$$\text{Extract yield by using hexane (\%)} = \frac{W_5}{W_6} \times 100 \quad [1]$$

Where

W_5 = net weight of extracts (grams)

W_6 = total weight of wood powder (grams)

Table 3-1 Weight of wood material for hexane extraction.

Extract number	Thimble and total weight (g)	Net Weight of wood powder (g)
1	W_{thimble} 2.4946	10.6182
	W_{total} 13.1128	

(Table 3.1 Con'd.)

2	W_{thimble}	2.9885	11.5813
	W_{total}	14.5698	
3	W_{thimble}	2.9505	11.8726
	W_{total}	14.8231	
4	W_{thimble}	2.9557	12.0213
	W_{total}	14.9770	
5	W_{thimble}	2.9042	11.696
	W_{total}	14.6002	
6	W_{thimble}	3.0354	10.9558
	W_{total}	13.9912	
7	W_{thimble}	2.9126	10.1828
	W_{total}	13.0954	
Total net weight of wood powder (g)			78.928

3.2.4 Soxhlet Extraction Using Ethyl Acetate

The soxhlet extraction chamber was initially rinsed using ethyl acetate. Then, the same extraction procedure as previously described for hexane was conducted to extract the solid residue except the temperature was 77 °C, which was the boiling point of ethyl acetate. The extract mixture was collected for the next steps. The following formula was used to determine the extract yield by using ethyl acetate. Table 3-2 shows the weight of wood material for ethyl acetate extracts.

$$\text{Extract yield by using ethyl acetate (\%)} = \frac{W_7}{W_8} \times 100 \quad [2]$$

Where

W_7 = net weight of extracts (g)

W_8 = total weight of wood powder (g)

Table 3-2 Weight of wood material for ethyl acetate extraction.

Extract number	Thimble and total weight (g)	Net Weight of wood powder (g)
1	W_{thimble}	3.4292
	W_{total}	14.2524

(Table 3.2 Con'd)

2	W_{thimble}	2.9396	
	W_{total}	13.6916	10.7520
3	W_{thimble}	3.0624	
	W_{total}	13.0907	10.0283
Total net weight of wood powder (g)			31.6035

3.2.5 Drying Using the Rotary Evaporator

The goal of this chapter was to analyze the components of POC heartwood. Therefore, during drying the temperature was as low as possible to protect any extracts from volatilizing. The drying system used for the concentration of the collected extract solutions was a rotary evaporator. The rotary evaporator system was comprised of a vacuum pump, controller, rotating evaporation flask, fluid bath, and a condenser with a flask. The rotating evaporation flask contained the mixture solution and was heated in the fluid bath. The solution boiled at a lower boiling point at lower pressure. So the solvent was removed without excessive heating which was helpful to keep the bioactivity of the extracts. The temperature of the water bath was set to 30 °C for the hexane extracts and 40 °C for ethyl acetate extracts. The dried samples of the two extracts were collected and stored at 4 °C for GC-MS analysis.

3.2.6 GC-MS Analysis

A Varian GC-MS (Gas chromatography-mass spectrometry) system was used to detect the extracts. The sample was eluted from the GC and was introduced into the ion trap Mass Spectrometer. Electron impact ionization (EI) was used as the ionization source. The vapor phase sample molecules were bombarded with high-energy electrons. As a result, the sample lost electrons after it was struck by high energy electrons and became a radical cation $M^{\bullet+}$. Many of the molecular ions were formed with enough excess energy to undergo subsequent reactions in the ionization chamber. Then, all ions with different m/z values went through the three ion trap electrodes and were trapped in a small volume. The fragments then went to the detector to record the m/z of different fragments and produce a MS spectrum.

The hexane and ethyl acetate extract went through the GC column and was eluted from the GC. Then, the separated components were introduced into the ion trap Mass Spectrometer. The column used for the Varian GC/MS system was a DB-5, and the carrier gas was helium. The initial temperature was 60 °C and was maintained for 1 min. The temperature then increased from 60 °C to 115 °C at a rate of 2.5 °C /min. The final temperature was 210 °C and was held for 30 min. The injector temperature was set at 250 °C, and the inject size was 1ml split 1:10. The MSD conditions had an electron impact source of 70eV and 250 °C.

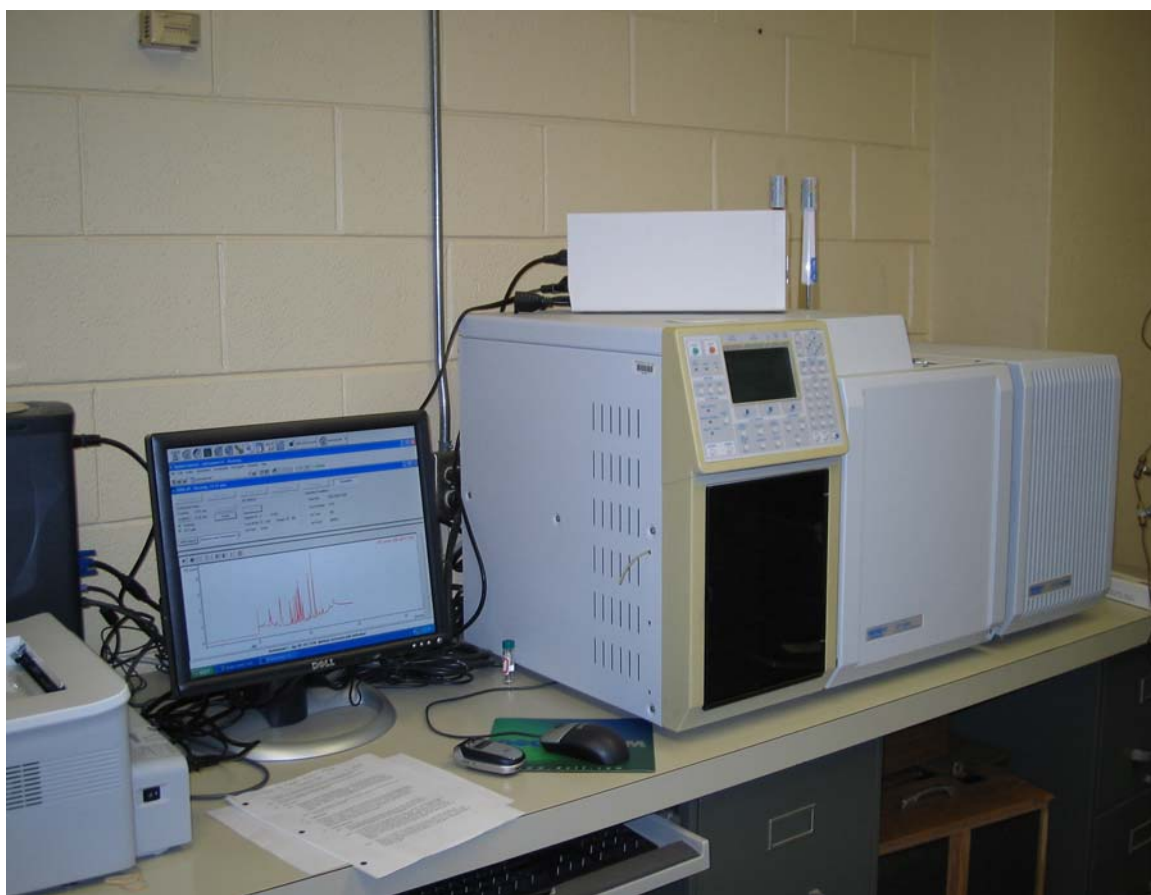


Figure 3-3. Varian GC-MS system

3.3. Results and Discussion

The results of the hexane and ethyl acetate extract yield are listed in Table 3-3 and 3-4. GC spectra are presented in Figure 3-4 (for n-hexane extracts) and Figure 3-5 (for ethyl

acetate extracts). Figure 3-6 presents mass fragments of sample and library standard of τ -Muurolol, and Figure 3-7 shows mass fragments for τ -cadinol. Relative percentage composition of hexane and ethyl acetate extracts are given in Table 3-5 and 3-6, respectively.

3.3.1 Hexane Extracts

Table 3-3 Weight of hexane extracts and extract yield (dry weight).

Item	Weight
Weight of vial (g)	13.1398
Total weight of vial and extracts (g)	14.3688
Net weight of hexane extracts (g)	1.2290
Net weight of wood powder (g)	78.9280
Extract yield (%)	1.557

3.3.2 Ethyl Acetate Extracts

Table 3-4 Weight of ethyl acetate extracts and extract yield (dry weight)

Item	Weight
Weight of vial (g)	13.1199
Total weight of vial and extracts (g)	13.5900
Net weight of hexane extracts (g)	0.4701
Net weight of wood powder (g)	31.6035
Extract yield (%)	1.4880

The objective of this portion of the study was to analyze the chemical components in the heartwood of POC and tried to find the compounds responsible for the special “ginger-like order.” During the grinding of the strips into wood powder, the operation temperature was set as low as possible to prevent any volatile compounds from volatilizing in the Wiley Mill. However, due to the heat produced by the high rotary rate of the mill, the temperature might be higher than 100°C. All of the extracts work was conducted with the wood powder produced by the Wiley Mill. So it was possible for some of the volatile components to come out and reduce the extracts yield. The Soxhlet

extract method was found to be effective. The solvent in the flask was heated to evaporate and moved up the condenser. Then, it was cooled down and dripped into the sample particles. Siphon action occurred which increased the extraction efficiency. After the Soxhlet extract, a rotary evaporator was used to dry the extracts. The temperature of the water bath was set to 30 °C for the hexane extracts and 40 °C for ethyl acetate extracts. At the operation temperature, all of the solvents were evaporated under low pressure. This study only analyzed the dry residual extracts obtained from the rotary evaporator.

The mixture solution produced by hexane extracting was almost colorless and oily with a light yellow color after concentrated by rotary evaporator. The mixture solution produced by ethyl acetate was also a yellow color and became a viscous oil with a light brown color after dried by the rotary evaporator. The yield of hexane extract was 1.557g/100g dry wood powder, and the yield for the ethyl acetate was 1.488g/100g dry wood powder.

3.3.3 GC-MS Spectrum

There are three significant peaks in the GC spectra of n-hexane extracts (Figure 3-4). Identification was made by retention time and National Institute of Standards and Technology (NIST) library search. The compounds τ -cadinol, azulene, and τ -muurolol were found in n-Hex extracts with the relative percentages of 35.7%, 9.0%, and 55.3%, respectively. The most abundant component (55.3%) in the n-hexane extract had a mass spectrum consistent with that of τ -muurolol, while the second most abundant (35.7%) was identified as τ -cadinol. In the GC spectra of EA extracts, more peaks were present (Figure 3-5) but only 4 could be clearly identified. Hexadecadinoic acid showed up at an earlier retention time (10.1 min.), followed by τ -cadinol, azulene, and τ -muurolol. The relative percentages of hexadecadinoic acid, τ -cadinol, azulene, and τ -muurolol in the ethyl acetate extracts were 16.8%, 26.2%, 7.0% and 50.0% respectively. Compared with the ethyl acetate extracts, n-hexane extracts have higher concentrations. The retention time of τ -cadinol, azulene, and τ -muurolol extracted from the heartwood of POC are 16.7, 16.8, and 17.0 min., respectively. Hexadecadinoic acid, τ -cadinol, azulene, and τ -muurolol were found in ethyl acetate extracts with the retention times of 10.1, 16.7, 16.8, and 17.0 min., respectively.

Date: 14 Apr 2006 16:32:18

Chromatogram Plot

File: c:\gcms\gaoheng\he-1.sms
Sample:
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Operator:
Date: 4/14/06 3:33 PM

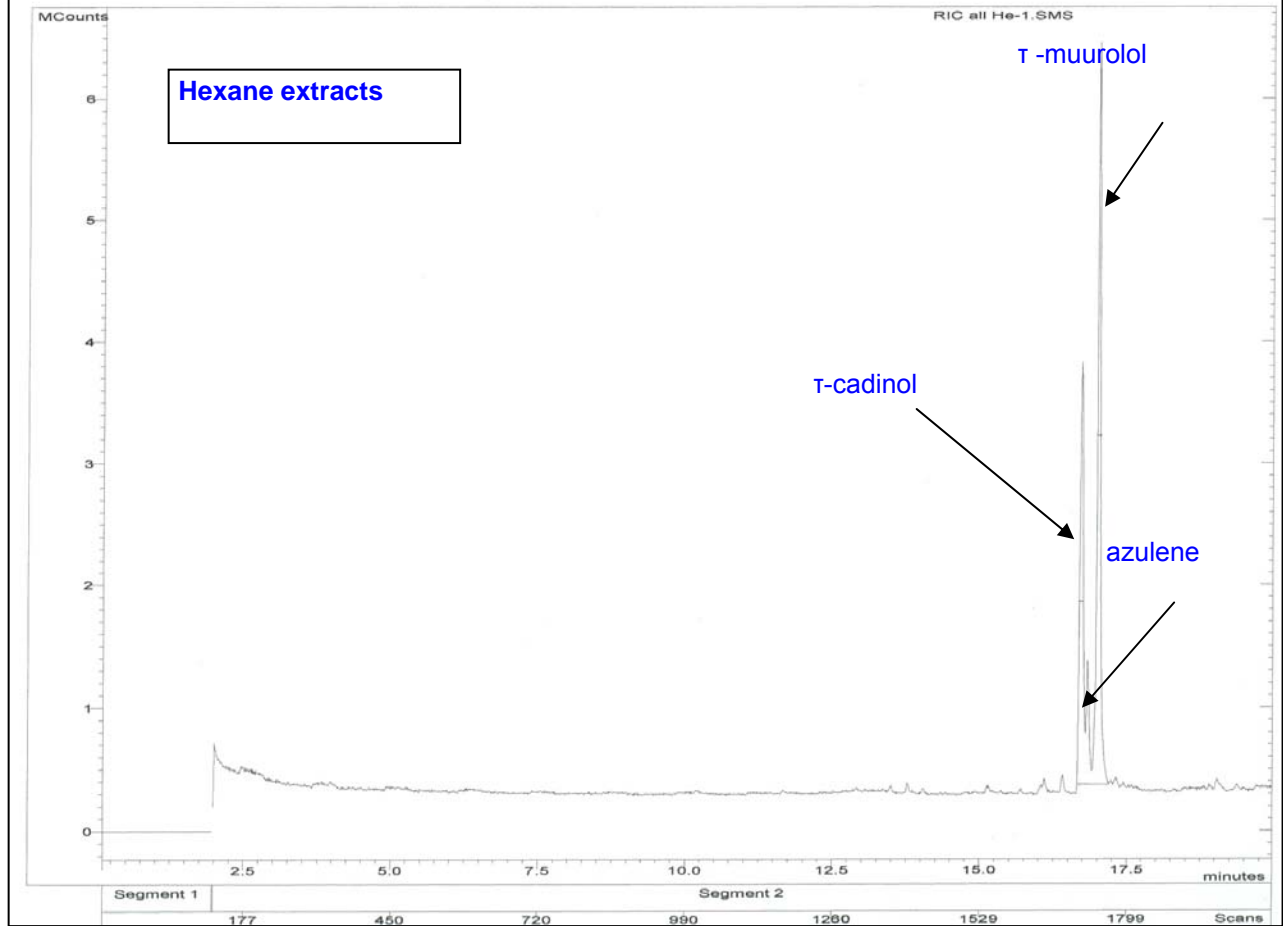


Figure 3-4. GC spectra of n-hexane extracts.

Print Date: 14 Apr 2006 16:40:56

Chromatogram Plot

File: c:\gcms\gaoheng\ea-1.sms
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Sample Notes: Manual injection with hydrogen carrier.

Operator: Operator
Date: 4/14/06 2:36 PM

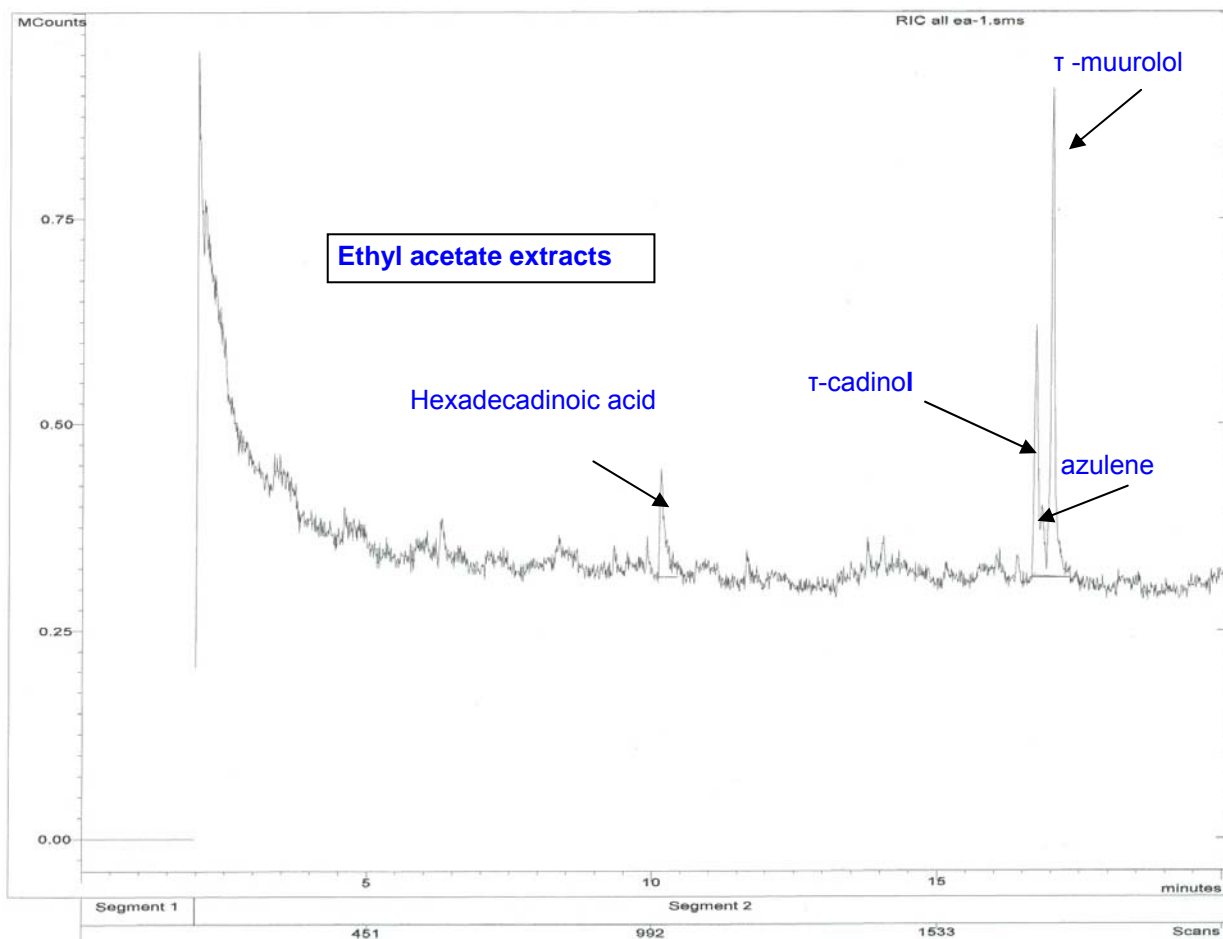


Figure 3-5. GC spectra of ethyl acetate extracts.

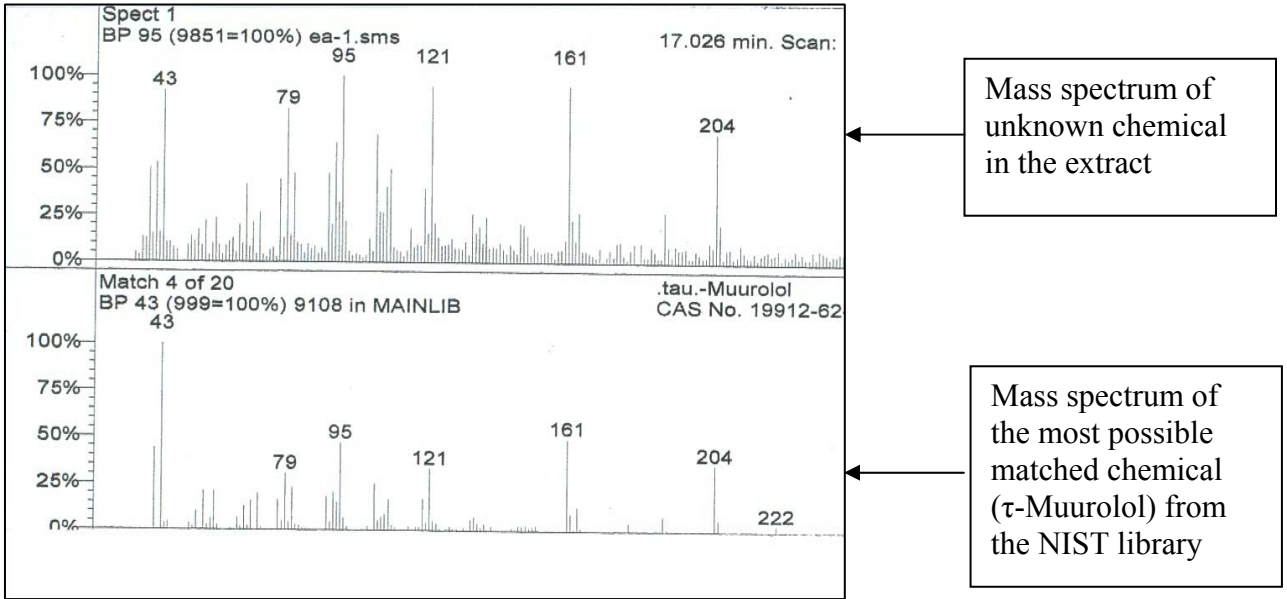


Figure 3-6. Mass fragments of unknown sample and library standard (τ -Muurolol).

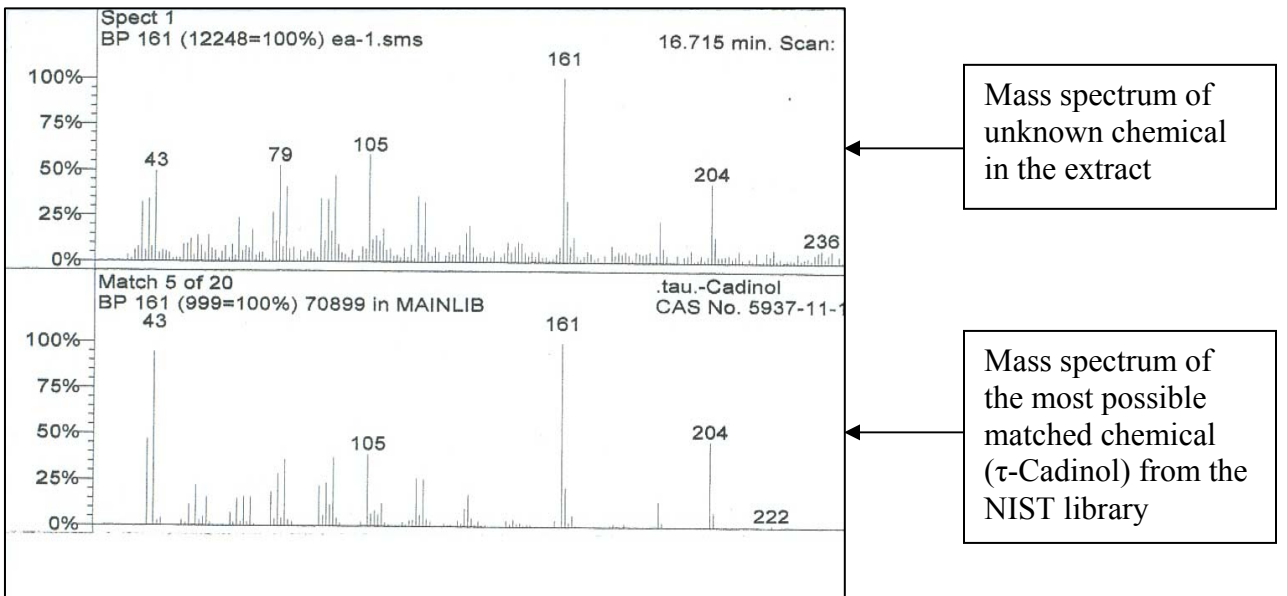


Figure 3-7. Mass fragments of unknown sample and library standard (τ -cadinol).

Table 3-5. Relative percentage composition of n-hexane extracts.

Constituents	Retention time (min.)	Relative percentage in extract (%)
τ -cadinol	16.7	35.7
azulene	16.8	9.0
τ -muurolol	17.0	55.3

Table 3-6. Relative percentage composition of ethyl acetate extracts.

Constituents	Retention time(min.)	Relative percentage in extract (%)
Hexadecadinoic acid	10.1	16.9
τ -cadinol	16.7	26.2
azulene	16.8	7.0
τ -muurolol	17.0	50.0

3.4. Summary

The Soxhlet extraction method was found to be effective to separate several chemicals from the “ginger-like order” heartwood. Ground wood samples were extracted by the Soxhlet method using hexane and ethyl acetate. The mixture solution produced by hexane extraction was almost colorless and appeared oily with a light yellow color after concentrated by the rotary evaporator. The mixture solution produced by ethyl acetate was also yellow in color and appeared as a viscous oil with light brown color after dried by the rotary evaporator. The results showed that the yields of hexane and ethyl acetate extracts were 1.557% and 1.448%, respectively. Based on the GC-MC results, τ -cadinol, azulene, and τ -muurolol were found in hexane extracts with the relative percentages of, 35.7%, 9.0%, and 55.3%, respectively. The retention time of τ -cadinol, azulene, and τ -muurolol extracted from the heartwood of POC were 16.7, 16.8, and 17.0 mins, respectively. Hexadecadinoic acid, τ -cadinol, azulene, and τ -muurolol were found in ethyl acetate extracts with the retention times of 10.1, 16.7, 16.8, and 17.0 minutes, respectively. The relative percentages of hexadecadinoic acid, τ -cadinol, azulene, and τ -muurolol were 16.8%, 26.2%, 7.0%, and 50.0% respectively.

3.5. References

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Chapter 4. Antifungal Analysis of Extracts of Heartwood of POC

4.1. Introduction

Solid and composite wood products are degraded by many organisms such as fungi and insects. The combined damage from Eastern and Formosan subterranean termites exceeds \$2 billion annually (Clausen and Yang 2004). Copper chromium arsenate (CCA), which accounted for about 80% of all treated timber in the USA in 1998, was highly effective in protecting wood against insect attack and fungal colonization and degradation. However, CCA is no longer available in the USA for residential uses and the use of CCA has been restricted in many European countries and Japan (Schultz and Nicholas 2002). Therefore, there is great interest and need to develop an environment-friendly wood preservative to replace traditional metal-based wood preservatives. One method to develop new metal-free wood preservatives is to first do extraction and identification work on naturally durable wood species and attempt to synthesize the compounds that are responsible for the inherent decay resistance.

Natural durability or decay resistance is the ability of wood to prevent biological degradation (Eaton and Hale 1993). Compared with sapwood, bark, and leaves, heartwood has higher natural decay resistance. Heartwood is located in the inner part of a tree stem, which has lower moisture content, usually darker color, and reduced permeability. A heterogeneous group of chemical compounds has been found in heartwood extractives, such as terpenoids, tropolones, flavonoids, stilbenes, and other aromatic compounds (Schffer and Cowiing 1966). The relationship between chemical composition in heartwood extractives and decay resistance was first reported by Hawley et al (1924). In a later study on North American conifers, an extract (pentane) of *Juniperus virginiana L.* was found to have an adverse effect on subterranean termites (Carter and Smythe 1974). Also, some researchers have investigated the relationship of extract structure against white- or brown-rot fungi resistance (Rudman 1963, Celimene et al 1999, Schultz et al. 1990). Debell et al. (1997) demonstrated that some cedar trees have a special bioactivity of decay resistance.

Port-Orford cedar (POC) is a unique tree species that has ginger-like odor and specific bioactivities. Previous work has shown that POC wood has excellent termite resistance (McDaniel 1989). Toxicity studies on Western juniper oil (*Juniperus*

occidentalis) and Port-Orford-Cedar oil (*Chamaecyparis lawsoniana*) extracts were conducted by utilizing local lymph node and acute dermal irritation assays (Craig 2004). Decay resistance activity has been reported by Morrell and Sexton (1987). However this research evaluated natural durability using soil-block testing according to ASTM standard D 2017-63 (Morrell and Sexton 1987), which can not separate and isolate the effective components in the wood. As a result, the compounds responsible for the decay resistance activity could not be identified. Therefore, to separate, isolate, and identify those effective components from the heartwood of Port-Orford cedar is a prelude for understanding and synthesizing environmentally-friendly wood preservatives.

In this chapter, I performed extract work on secondary metabolites of the heartwood of POC by sequentially extracting with hexane and ethyl acetate. The antifungal activities were tested with, brown-rot fungi (*Gloeophyllum trabeum*) and white-rot fungi (*Trametes versicolor*). The antifungal index (AI) was tested to indicate the performance of the antifungal activities.

4.2. Materials and Methods

4.2.1 Chemicals

Hexane and ethyl acetate used for the extract work were standard analytical grade. Malt extract, agar, and yeast extract were used for the antifungal test.

4.2.2 Preparation of Extract

The heartwood of POC was collected from a 10 cm-thick disk of a log and stored at -4°C.

The sample was reduced to small particles in a Wiley Mill. The particles selected for analyses passed through a 40-mesh screen. Ground wood samples were Soxhlet extracted. A lower polarity solvent, n-hexane, was used for the first step, and the higher polarity ethyl acetate was used for the second extraction step. The extract solutions were dried in a rotary evaporator denoted as n-hexane and ethyl acetate to represent n-hexane and ethyl acetate, respectively. The yields of n-hexane and ethyl acetate extracts were 1.557% and 1.448%, respectively.

4.2.3 Evaluation of antifungal activities

The antifungal activities were evaluated according to Chang et al. (1999) with slight modifications. A medium was prepared by using 2% malt extract, 1.5% agar, and 0.005% yeast extract and sterilized for 20 min. The n-hexane and ethyl acetate extract solutions

were prepared with a concentration range of 0.125-2.5 mg·mL⁻¹ to mix with the culture medium. White-rot and brown-rot fungi were transferred to the center of the Petri dishes and incubated at room conditions. Two controls were included: one control contained the culture media only and second control contained the solvent only. When the fungi reached the edges of the control Petri dishes, the diameter was measured and the antifungal index (AI) was expressed as % inhibition, which was calculated by the following formula. This estimation of antifungal activities was carried out in triplicate and the results were averaged.

$$\%AI = \left(\frac{D_2 - D_1}{D_2} \right) \times 100 \quad [1]$$

Where

D_2 = diameter growth in the control dishes (mm)

D_1 = diameter growth in the experimental Petri dishes with extracts (mm).

4.3. Results and Discussion

Figure 4-1 and 4-2 shows the brown-rot fungi inhibitory effects of n-hexane and ethyl acetate extracts and Figure 4-3 and 4-4 present the white-rot fungi inhibitor effects. The anti fungal index (AI) of the two extracts is presented in Figure 4-5. Figure 4-6 and 4-7 present the antifungi activity comparison between n-hexane and ethyl acetate extracts.

4.3.1 Effect of Anti Brown-rot Fungi Activity

It took the brown rot fungi 7 days to reach the edges of the control dishes. As for n-hexane extracts (Figure 4-1), there were 7 Petri dishes with different growth diameters and the concentration of the n-He samples from left to right were 0 (control), 0.05, 0.125, 0.25, 0.5, 1.25, and 2.5 mg/ml. As for ethyl acetate extracts (Figure 4-2), due to their lower activities one more concentration (5 mg/ml) was used to evaluate the activity. So the total concentration series of the ethyl acetate extracts sample from left to right were 0 (control), 0.05, 0.125, 0.25, 0.5, 1.25, 2.5, and 5 mg/ml. The more effective inhibitors of fungi growth resulted in larger zones of clearance. Therefore, both of the extracts have growth inhibitory effect on brown-rot fungi.

4.3.2 Effect of Anti White-rot Fungi Activity

Because the white-rot fungi grew slower than the brown-rot fungi, it took 14 days for the white-rot fungi to reach the edges of the control dishes. The concentrations of the n-

The sample from left to right are 0 (control), 0.05, 0.125, 0.25, 0.5, 1.25, and 2.5 mg/ml (Figure 4-3) and the total concentration series of the EA sample are 0 (control), 0.05, 0.125, 0.25, 0.5, 1.25, 2.5, and 5 mg/ml (Figure 4-4). According to Figure 4-3 and 4-4, the Petri dishes with different sample concentrations have different growth diameters and different clearances. Therefore, both of the extracts had a growth inhibitory effect on white-rot fungi.

4.3.3 Comparison Between n-hexane and Ethyl Acetate Extracts

In Figure 4-6, the top row is ethyl acetate extracts, and the bottom row is n-hexane extracts, both of which have the concentrations of 2.5, 1.25, 0.5, 0.25, and, 0.125 mg/ml (from left to right). In Figure 4-7, the Petri dishes with n-hexane extracts are in the top row and those with ethyl acetate extracts are in the bottom row. The concentration of the samples (from left to right) are 0.125, 0.25, 0.5, 1.25, and 2.5 mg/ml. Therefore, both the brown-rot and white-rot fungi were susceptible to inhibition by n-hexane and ethyl acetate extracts. Because n-hexane extracts had larger zones of clearance at the same concentration, it had a stronger inhibition of white- and brown-rot fungi.

The fungal inhibition properties of the two extracts of POC exhibited concentration-dependent activity. The n-hexane extracts showed a higher antifungal index in this assay system with white- and brown-rot inhibition percentages of 83.27 and 68.60 %, respectively, which were significantly higher than the ethyl acetate extracts (77.82 and 56.20 %, respectively) at a concentration of 2.5 mg·mL⁻¹.

4.4. Summary

There is a great interest to identify safe and natural compounds to replace traditional metal-based wood preservatives. Antifungi tests showed that both the brown-rot and white-rot fungi were susceptible to inhibition by n-He and EA extracts and n-He extracts had a stronger inhibition of white-rot and brown-rot fungi. The antifungal index (AI) is an effective quantitative method to evaluate bioactivity.

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Figure 4-1. Anti-brown-rot fungi activity of n-hexane extracts.



Figure 4-2. Anti-brown-rot fungi activity of ethyl acetate extracts.

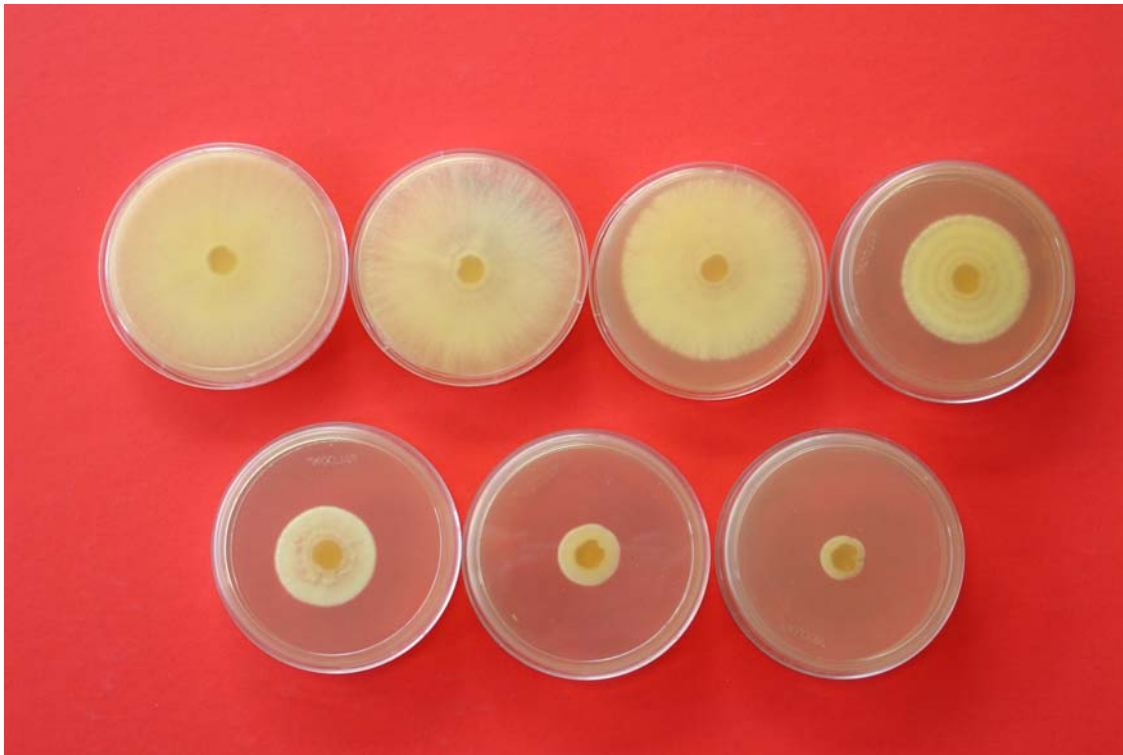


Figure 4-3. Anti-white-rot fungi activity of n-hexane extracts.



Figure 4-4. Anti-white-rot fungi activity of ethyl acetate extracts.

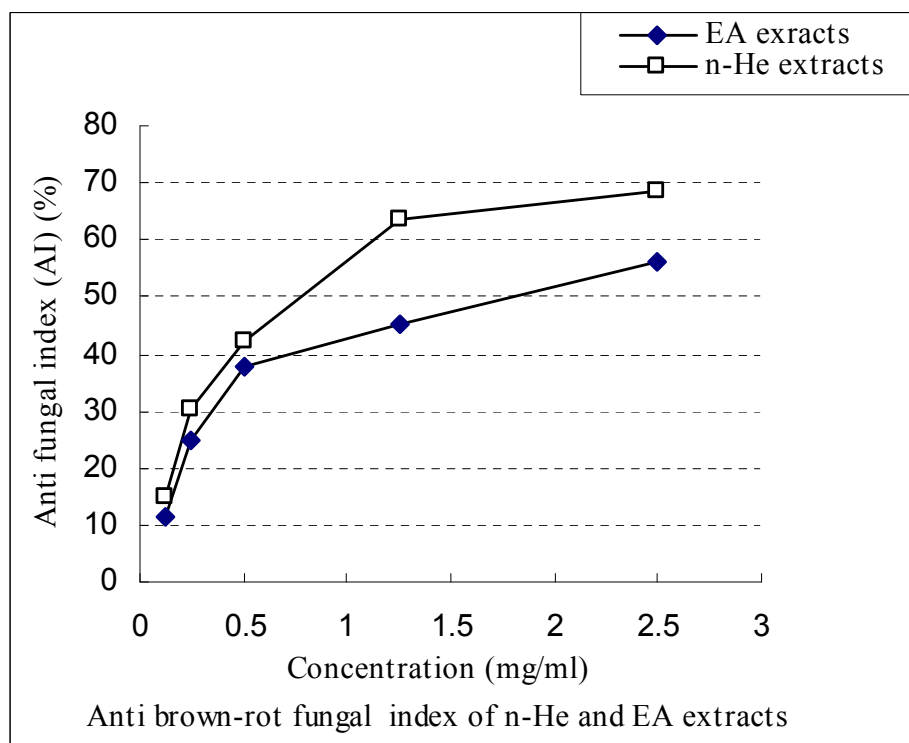
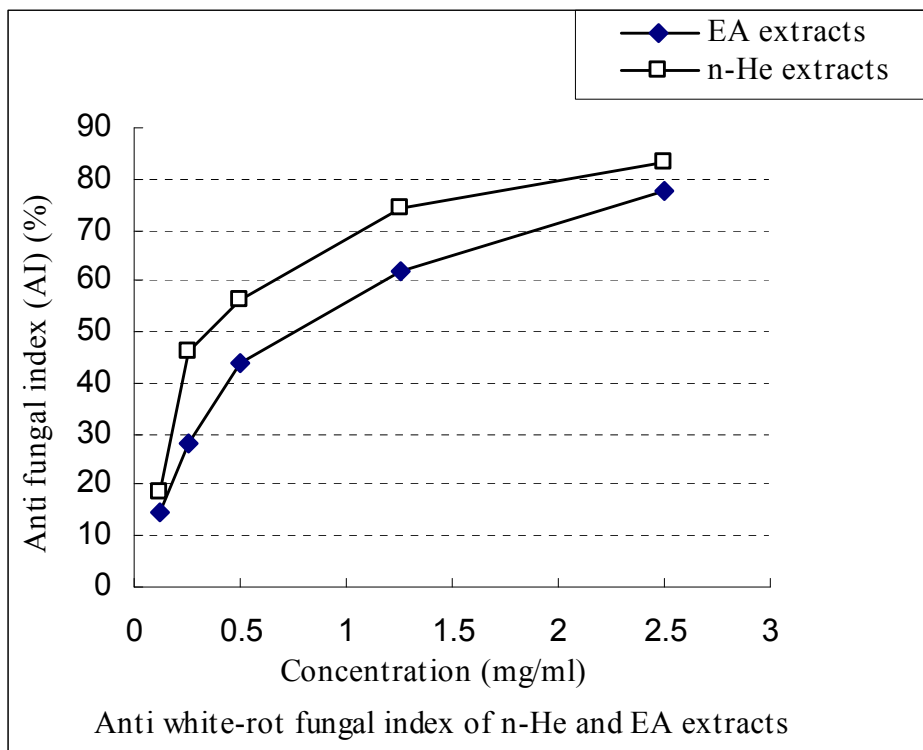


Figure 4-5. Anti fungal index of n-hexane and ethyl acetate extracts.



Figure 4-6 Anti-white-rot fungi effects between n-hexane and ethyl acetate extracts. The top row is ethyl acetate extracts, and the bottom row is n-hexane extracts.

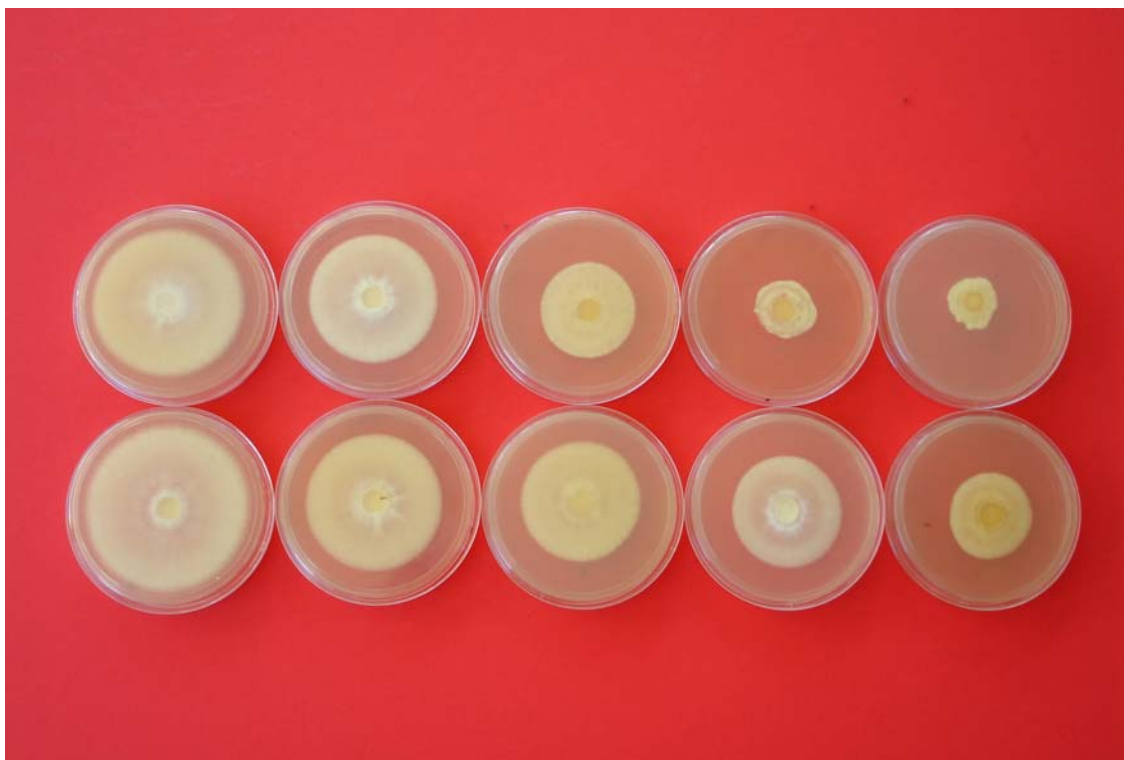


Figure 4-7 Anti-brown-rot fungi effects between n-hexane and ethyl acetate extracts. The top row is n-hexane extracts, and the bottom row is ethyl acetate extracts.

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Chapter 5 Conclusions

There are several conclusions that can be derived from this study:

1. Soxhlet extraction is an effective method to separate antioxidant components from heartwood, sapwood, inner bark, and outer bark. The methanol extracts of heartwood were yellow and oily; sapwood extracts were a light yellow powder with the lowest yield (1.0%). Inner bark extracts showed a red color with the highest yield (16.5%), and the bark extracts were a dark red powder also with a high yield (15.9%).
2. N-hexane, ethyl acetate, and n-Butanol were effective for advanced separation of bark based on different polarity. Four subtractions (n-He, EA, n-Bu, and Wa) produced yields of 5.077, 7.739, 2.421, and 0.6423 (w/w), respectively.
3. In order to analyze the special “ginger-like order” compounds of the heartwood of POC, samples were successively Soxhlet extracted using different polar solvents. A low polarity solvent, hexane, was used for the extraction in the first step, and then the higher polarity ethyl acetate was used to do the second extraction step. Two different components were found, n-hexane and ethyl acetate extracts, with yields of 1.557 and 1.4880%, respectively.
4. In the DPPH• scavenging assay, all of the heartwood, sapwood, inner bark, and outer bark exhibited concentration-dependent DPPH• radical scavenging activities. The heartwood and the sapwood revealed moderate antioxidant activities and the inner bark and outer bark extracts showed the most activities. Also, the advanced subtraction of bark showed different DPPH• scavenging abilities. The IC₅₀ values of n-He, EA, n-Bu, and Wa subtractions were 115.4 μg·mL⁻¹, 13.04 μg·mL⁻¹, 6.53 μg·mL⁻¹, and 22.48 μg·mL⁻¹, respectively.
5. In the ABTS⁺• radical cation scavenging assay, inner bark showed an inhibition effect on ABTS⁺• at all test concentrations that were higher than the synthetic antioxidant, BHT, in the same experimental condition, followed by outer bark and sapwood extracts. The heartwood extract showed the weakest radical-scavenging ability. Among the four partitions of POC bark, n-Bu showed the highest inhibition effect on ABTS⁺• followed by EA, Wa, and n-He subtractions.

6. In the metal chelating activity assay, sapwood extracts demonstrated the strongest chelating metal capability. The heartwood, outer bark, and inner bark showed negligible metal chelating activity.

7. The inner bark contained the highest amount of total phenolic compounds, 537.5 mg GAE per g of extract, followed by outer bark, sapwood, and heartwood. N-Bu contained the highest amount, 428.5.5 mg GAE per g of extract, followed by EA, Wa, and n-Bu. The correlation suggested that phenolic compounds are likely to contribute to the radical scavenging activity of the methanol extracts.

8. τ -cadinol, azulene, and τ -muurolol were found in n-hexane extracts with the relative percentages of, 35.7%, 9.0%, and 55.3%, respectively. The retention time of these three main compounds extracted from the heartwood of POC was 16.7, 16.8, and 17.0 min., respectively. Hexadecadinoic acid, τ -cadinol, azulene, and τ -muurolol were found in ethyl acetate extracts with the retention times of 10.1, 16.7, 16.8, and 17.0 min., respectively. The relative percentages of ethyl acetate extracts, hexadecadinoic acid, τ -cadinol, azulene, and τ -muurolol were 16.8%, 26.2%, 7.0% and 50.0%, respectively.

9. Both the n-hexane and ethyl acetate extracts had growth inhibitory effects on white- and brown-rot fungi. Because n-hexane extracts had larger zones of clearance at the same concentration, it had a stronger inhibition of white-rot and brown-rot fungi. The fungal inhibition properties of the two extracts of POC exhibited concentration-dependent activity. The n-hexane extracts showed a higher antifungal index in this assay system with white- and brown- rot inhibition percentages of 83.27 and 68.60 %, respectively, which were significantly higher than the ethyl acetate extracts (77.82 and 56.20 %) at a concentration of $2.5 \text{ mg}\cdot\text{mL}^{-1}$.

Vita

The author was born in Shandong, China, on April 8, 1977. She earned the degree of Bachelor of Science from the Department of Chemistry at Qingdao University in 2001, and then she went to the Graduate School of Beijing Institute of Technology (BIT) majoring in environmental engineering from 2001 to 2004. She received a Master of Engineering degree in environmental engineering from BIT in 2004. From May, 2004, to December, 2004, she worked as an environmental engineer at Beijing SPC Environmental Protection Tech Engineering Co., Ltd, P.R. China. The author then joined the wood science program at Louisiana State University as a master. student under the supervision of Dr. Todd F. Shupe in January, 2005. The author's study focused on the chemical analysis of extracts from Port-orford cedar wood and bark.