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Antioxidant activity of extracts from the wood and bark of Port Orford cedar

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Abstract Heartwood, sapwood, and inner and outer bark of Port Orford cedar were extracted with methanol, and the extracts evaluated for antioxidant activity. The total phenol content (TPC) of the extracts was determined by the Folin-Ciocalteu method and expressed as gallic acid equivalent (GAE). Butylated hydroxytoluene was used as a positive control in the free-radical-scavenging activity tests and ethylenediaminetetraacetic acid dihydrate disodium salt served as a positive control in the metal-chelating activity assay. All wood extracts showed significant free-radical-scavenging activity. In the radical-scavenging assay of 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (the ABTS assay), the inner bark extracts exhibited the strongest free-radical-scavenging activity. The 50% inhibitory concentrations (IC₅₀) in the radical-scavenging assay against 1,1-diphenyl-2-picrylhydrazyl hydrate radical (DPPH) of the heartwood, sapwood, and inner and outer bark extracts were 64.77, 29.03, 10.31, 19.87 $\mu\text{g}\cdot\text{ml}^{-1}$, respectively. In the metal-chelating activity system, the sapwood extract demonstrated significant activity. The greatest TPC, 537.5 mg GAE/g dry extract, was detected in the inner bark. The lowest TPC of 136.9 mg GAE/g dry extract was observed in the heartwood dry extract. The results indicate that the antioxidant activities of the extracts are in accordance with the amounts of phenolics present; the inner and outer barks of Port Orford cedar are rich in phenolics and may provide good sources of antioxidants.

Key words Free radical scavenger · Methanol extracts · Port Orford cedar · Total phenol content

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Introduction

Port Orford cedar (POC) is a large tree, reaching 43–55 m in height and 1.2–1.8 m in diameter, and is restricted to the coastal forests of southwestern Oregon and northern California in the United States.¹ Botanically, it is known as *Chamaecyparis lawsoniana* (A. Murr.) Parl., 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 exhibiting bioactivity such as antioxidant and antimicrobial properties.^{2–4} Previous work has shown that POC wood has excellent decay and termite resistance.^{5–8}

Free radicals and reactive oxygen species (ROS) are byproducts arising from numerous physiological and biochemical processes. Some research reports have shown that excessive ROS may be harmful to biomolecules and promote aging,⁹ cancer,¹⁰ and cardiovascular diseases.¹¹ Consequently, protecting organisms from oxidative damage by use of antioxidants is one approach to prevent these diseases. However, previous research has reported that some synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), are carcinogenic.^{12–14} Hence, there is a great desire to search for safe and natural antioxidants as nutritional supplements and health food.

Another impetus to investigate the antioxidant activity of POC is related to recent developments in the wood preservation industry. In the United States, copper chromium arsenate (CCA) can no longer be used to treat wood products for residential uses. In several European countries, CCA and other metal-based preservatives have been banned for all types of use for several years now. As an alternative, antioxidants are being studied as an option to develop environmentally benign wood preservatives. Recent reports have suggested that free radical species may help to disrupt the cell walls of wood and thus facilitate the penetration of white- and brown-rot fungal enzymes.^{15–17}

Accordingly, the free-radical-scavenging properties of methanol extracts from some decay-resistant tree species may inhibit this early step in the decay process. Recent studies have shown that the combination of antioxidants and an organic biocide gives wood enhanced protection against fungal decay.^{18,19}

Despite the considerable bioactivity of POC, there are few, if any, detailed studies on the antioxidant properties of this species. Based on the inherent decay and termite resistance of POC, further research to investigate the potential antioxidant activity of extractives from POC wood is prudent. Therefore, the objective of this study was to extract the antioxidants and evaluate their activity. Several *in vitro* assays, including 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical-scavenging assay and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation-scavenging ability assay, were carried out to evaluate the antioxidant activity of POC. Also, the metal-chelating activity was determined in this study by a ferrozine-Fe²⁺ reaction system.

A previous study found that brown sugar aqueous solutions exhibited weak free-radical-scavenging activity in the DPPH assay and antioxidant activity in the ABTS assay at relatively high concentration. Moreover, the brown sugar extracts showed interesting free-radical-scavenging properties despite the low concentration of phenolic and volatile compounds.²⁰ Previous research has shown that phenolic compounds extracted from plants are potential antioxidants.²¹ Hence, we quantitatively analyzed the total phenol content in wood and bark of POC by Folin-Ciocalteu assay.

Materials and methods

Chemicals

Potassium persulfate, ABTS, and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid (ferrozine) as its sodium salt, were purchased from Sigma (St. Louis, MO, USA); ferrous chloride, gallic acid, DPPH, and ethylenediaminetetraacetic acid dihydrate disodium salt (EDTA-Na₂·2H₂O) were purchased from Alfa Aesar (Ward Hill, MA, USA); and butylated hydroxytoluene (BHT) was purchased from Alfa Aesar (Lancaster, UK). All of the other chemicals were of standard analytical grade except the methanol used in the DPPH radical assay (HPLC grade), and the ethanol used in the ABTS test (spectrophotometric grade). The final concentrations of the extracts (heartwood, sapwood, inner bark, outer bark) and BHT in the DPPH and ABTS assays were 1.765, 3.529, 5.882, 11.76, and 29.41 μg·ml⁻¹ in the reaction mixtures and the total volume was 5.1 ml for each reaction mixture. In the analysis of metal-chelating activity, the final concentrations of the extracts (heartwood, sapwood, inner bark, outer bark) and the positive reference were 17.14, 34.28, 57.14, 114.29, and 285.71 μg·ml⁻¹ in the reaction mixtures.

Preparation of extracts

Inner bark, outer bark, sapwood, and heartwood of POC were collected from a 10-cm-thick disk that was cut approximately 12 cm from the bottom end of a saw log and stored at -4°C. The samples were initially air-dried and then reduced to small particles in a Wiley mill. The particles selected for analyses passed through a 40-mesh screen. Soxhlet extraction of 100 g of particles was conducted with methanol (1 liter) until the solvent became colorless. Then all of the extracts were collected, taken to dryness in a rotary evaporator, lyophilized, and kept in darkness at 4°C until testing. Extract yields of heartwood, sapwood, inner bark, and outer bark of POC were 3.9%, 1.0%, 16.5%, and 15.9% (w/w), respectively.

Evaluation of antioxidant activities

DPPH radical-scavenging assay

Radical-scavenging activity of POC against stable DPPH was determined spectrophotometrically according to Burits and Bucar²² and Cuendet et al.²³ Methanol solutions (0.1 ml) of the extract at various concentrations were added to 5 ml of a methanol solution of DPPH or methanol alone (blank). The reaction mixture was vigorously shaken by hand and then kept in darkness for 30 min at ambient conditions. The absorbance was measured at 512 nm, and the antioxidant capacity was expressed as percent inhibition, which was calculated by Eq. 1 in which A₀ equals absorbance without extracts and A₁ equals absorbance with extracts at 512 nm.

$$\% \text{Inhibition} = \left(\frac{A_0 - A_1}{A_0} \right) \times 100 \quad (1)$$

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 the 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 experiment was performed according to an improved method as described by Re et al.²⁴ with some modification. ABTS was generated by mixing 5 ml of 7 mM ABTS with 88 μl of 140 mM potassium persulfate under darkness at room temperature (23°C) for 16 h. The solution was diluted with 50% ethanol and the absorbance at 734 nm was measured. The ABTS radical cation-scavenging activity was assessed by mixing 5 ml ABTS solution (absorbance of 0.7 ± 0.05) with 0.1 ml POC extract or negative control (methanol). The final absorbance was measured at 734 nm. The inhibition percentage of ABTS was calculated using

Eq. 1 with some modifications. A_0 equals the absorbance without extracts at 734 nm; A_1 equals the absorbance with extract or BHT at 734 nm. All experiments were performed in triplicate.

Analysis of metal-chelating activity

The metal chelating activity of extracts was estimated according to Dinis et al.²⁵ In short, 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 was kept at room temperature. The absorbance was then read at 562 nm after the reaction mixture reached equilibrium in 10 min. $\text{EDTA-Na}_2 \cdot 2\text{H}_2\text{O}$ served as a positive control, and a sample without extract and $\text{EDTA-Na}_2 \cdot 2\text{H}_2\text{O}$ served as a negative control. Triplicate samples were run for each set and the results averaged. The percentage inhibition of ferrozine- Fe^{2+} complex formation was calculated based on Eq. 1 in which A_0 equals absorbance without extracts and A_1 equals absorbance with extracts at 562 nm.

Determination of total phenolic content

Total phenolic content (TPC) of methanol crude extracts was determined according to the Folin-Ciocalteu method²⁶ with slight modification. The result was expressed as gallic acid equivalents (GAE), which indicates the phenolic content as the amount of gallic acid in milligrams per gram of dry weight of a sample. Methanol solutions of the extracts (0.5 ml) were mixed with tenfold diluted Folic-Ciocalteu reagent (2.5 ml) and incubated for 2 min at room temperature before the addition of sodium carbonate solution (2 ml, 7.5% w/v). The absorbance of the mixture solution was measured at 765 nm after standing for 30 min 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. This estimation of phenolic compounds in the extracts was conducted in triplicate, and the results were averaged.

Results

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 DPPH in methanol solution in the presence of a hydrogen-donating antioxidant due to the formation of the nonradical form DPPH-H.^{25,26} This transformation results in a color change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple color is monitored at 517 nm. The radical-scavenging properties of the various extracts of POC are given in Fig. 1. All POC extracts exhibited concentration-dependent DPPH radical-scavenging activity. Among the extracts isolated, the

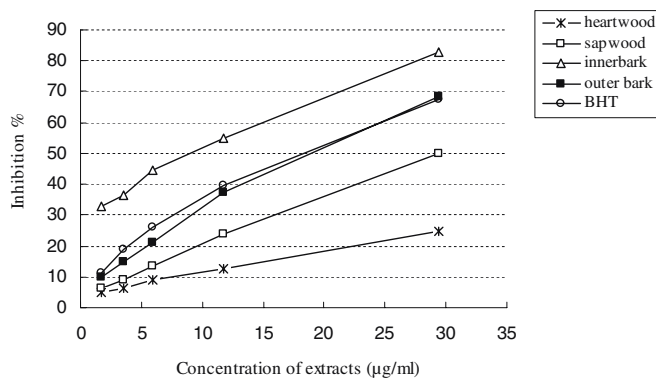


Fig. 1. Radical scavenging ability of the extracts of Port Orford cedar (POC) and butylated hydroxytoluene (BHT; as positive control) against 1,1-diphenyl-2-picrylhydrazyl hydrate radical (DPPH)

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 IC_{50} values of heartwood, sapwood, inner bark, and outer bark extracts were 64.77, 29.03, 10.31, and 19.87 $\mu\text{g}\cdot\text{ml}^{-1}$, respectively. The IC_{50} value of the reference compound, BHT, was approximately 19.36 $\mu\text{g}\cdot\text{ml}^{-1}$. The inner bark showed the lowest IC_{50} , which indicated it was the most effective against DPPH radical, including comparison with the well-known antioxidant BHT. The IC_{50} value of heartwood was obtained by extrapolation because of its low antioxidant activity.

Effect of ABTS radical cation-scavenging activity

ABTS radical cation decolorization 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 ABTS and potassium persulfate directly generates the blue/green ABTS chromophore, which can be reduced by an antioxidant, thereby resulting in a loss of absorbance at 734 nm. The method of Re et al.²⁴ was applied in this study to obtain the radical-scavenging data shown in Fig. 2. The control, BHT, and the four extracts exhibited concentration-dependent ABTS radical-scavenging activity. At concentrations from 1.765 to 5.882 $\mu\text{g}\cdot\text{ml}^{-1}$, the slopes of the curves of inhibition percentage versus concentration for inner bark and BHT were larger than those for the other three extracts and demonstrate that in that concentration range the antiradical 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 ABTS 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 ABTS at all test concentrations that

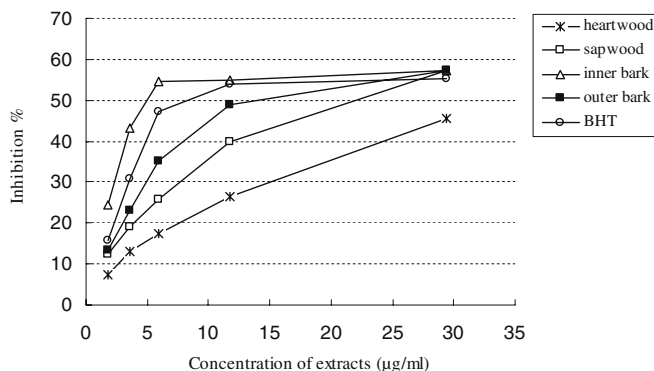


Fig. 2. Radical scavenging ability of BHT and the extracts of POC against 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical (ABTS)

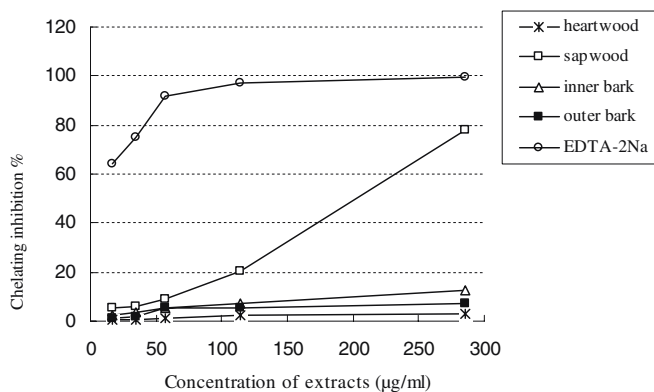


Fig. 3. Metal-chelating capability of the extracts of POC, with ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) as positive control

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.

Metal-chelating activity

Ferrozine can quantitatively chelate with Fe^{2+} and forms a complex with a red color. This reaction is limited in the presence of other chelating agents and results in a decrease 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.²⁷ The chelating abilities of samples and EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$ are given in Fig. 3. It should be noted that of the four samples, the sapwood extract demonstrated the strongest metal-chelating capability. The heartwood, outer bark, and inner bark extracts showed negligible metal-chelating activities.

Total phenol content of extracts

The content of phenolics in methanol extracts was determined from a regression equation of the calibration curve

Table 1. Total phenol content (TPC) of extracts from wood and bark of Port Orford cedar

Sample	TPC in dry extract (mg GAE/g)	TPC in dry wood powder (mg GAE/g)
Heartwood	136.9	5.353
Sapwood	257.7	2.559
Inner bark	537.5	88.76
Outer bark	489.1	77.65

GAE, gallic acid equivalent

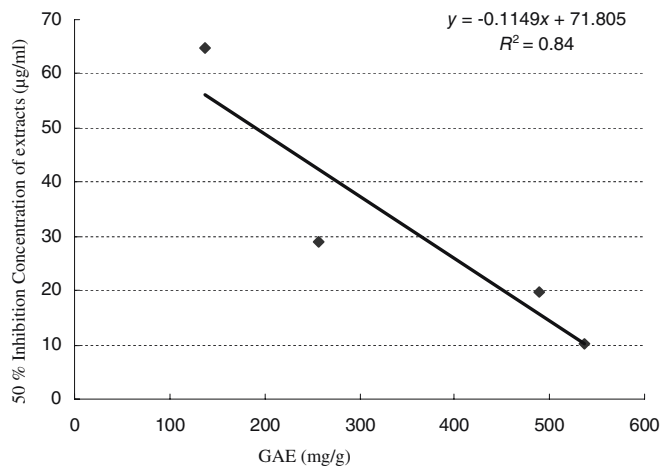


Fig. 4. Correlation of total phenol content (expressed as gallic acid equivalent; GAE) and 50% inhibitory concentration (IC_{50}) values from the DPPH scavenging test

($y = 0.004663x + 0.0565$, $R^2 = 0.99$) and was expressed in gallic acid equivalents (GAE). As can be seen from Table 1, the distribution of phenolic compounds in the four extracts from POC demonstrated that the inner bark contained the highest amount, 537.5 mg GAE/g extract, followed by the outer bark, sapwood and heartwood. However, the phenolic content of the sapwood (GAE per gram dry wood powder) showed the lowest value because it had the lowest extract yield (0.9931%, w/w) among the four samples. Figure 4 shows that the phenolic content in the extracts correlate well with the IC_{50} of the DPPH radical-scavenging assay. The correlation suggests that phenolic compounds are likely to contribute to the radical-scavenging activity of the methanol extracts.

Discussion

Antioxidant properties and other bioactivities of secondary metabolites of plants are of great interest in many fields such as pharmacology and the food nutrition industry. It is a growing tendency that natural antioxidant compounds are being used to replace synthetic antioxidants due to their side effects. This study is the first to evaluate the antioxidant activity of various tissue types of POC in a comprehensive in vitro method, as well as their total phenolic content by the Folin-Ciocalteu test. Results showed that methanol ex-

tracts exhibited promising radical-scavenging activity. More specifically, the inner bark extract 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 compounds contained in the POC that can reduce the DPPH and ABTS free radicals.

Transition metal ions may act as catalysts that promote the generation of radicals, thereby initiating oxidative chain reactions, while the chelating agents, which form σ -bonds with the metals, can stabilize the oxidized form of the metal ion and then decrease the possibility of oxidative chain reactions.²⁸ The present study detected significant chelating activity against Fe^{2+} in the sapwood extract. This may be because sapwood is a living tissue 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 that have high chelating capability. This may infer high potential development for biological or food systems in order to safeguard human health, and food quality, stability, and safety.

Extracts from wood and bark are complex natural products. Generally, the inner bark and sapwood are rich in nutrients such as sucrose and glycosides. Heartwood and outer bark, by contrast, are typically deficient in these nutrients, but are rich in secondary metabolites such as the tannins and other phenolics that function to protect the living tissues against biological attack. Because the outer bark is essentially nonliving, the inner bark provides the first active line of defense for a tree against oxidation. We attribute the highest level of antioxidant activity in the inner bark to this critical function. As the inner bark ceases to function, it forms new layers of outer bark that would likely contain any remaining antioxidant compounds from the inner bark, albeit at somewhat lower levels. Recognizing that the sapwood is actively involved in transporting inorganic nutrients from the roots to the rest of the tree, it seems logical that this tissue would have the highest metal-chelating capacity. Results from this study demonstrate differences in antioxidant and metal-chelating capacity among the POC tissues analyzed. Studies are in progress to isolate and identify the chemical compounds that contribute to the total antiradical activities and the potential metal-chelating activity to better understand their mechanism of action as antioxidants. Future research on the relationship between antioxidants and decay resistance and on application possibilities is needed.

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