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Antioxidant activity of extracts from the bark of *Chamaecyparis lawsoniana* (A. Murray) Parl.

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Abstract

The bark of *Chamaecyparis lawsoniana* (A. Murray) Parl. was extracted with methanol and sequentially partitioned with *n*-hexane, ethyl acetate, *n*-butanol and deionized water. The antioxidant activities of the four extracts were evaluated using the DPPH• and $ABTS^{+\bullet}$ methods. The total phenolic content of the extracts was determined using the Folin-Ciocalteu method and expressed as gallic acid equivalents (GAE). Butylated hydroxytoluene was used as a positive control in the radical-scavenging activity tests. All the bark extracts showed significant radical-scavenging activity. In the $ABTS^{+\bullet}$ assay, *n*-butanol extracts exhibited the strongest radical-scavenging activity, followed by ethyl acetate, water, and *n*-hexane extracts. The greatest total phenolic content was 428.54 mg GAE per gram of dry extract and was detected in the *n*-butanol extract, followed by the ethyl acetate and *n*-hexane extracts. The antioxidant activities correlate with the amount of phenolics present in these extracts. The ethyl acetate and *n*-butanol extracts were rich in phenolics and may represent a good source of antioxidants.

Keywords: antioxidant activity; Folin-Ciocalteu method; free radical scavenger; methanol extracts; Port-Orford cedar; total phenolic content.

Introduction

Free radicals and reactive oxygen species are byproducts of numerous physiological and biochemical processes. Polyphenol compounds are reported to be a good source of 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. Besides the traditional resources used for antioxidants, many plant species have been investigated in the search for natural antioxidants (Banias et al. 1992). The discovery of taxol in the bark of the Pacific yew tree stimulated interest in antioxidants from woody plants and other medicinal plants as anticancer agents. Compared with wood or leaves, bark is the most economical and convenient resource for the extraction of possible antioxidant compounds. For southern pines of pulpwood size, bark volumes usually range between 12% and 24% and are negatively correlated with tree diameter (Koch 1972). However, the primary use of bark is currently to burn it as fuel, in spite of its low fuel value (Karchesy and Koch 1979). Previous studies have focused on the isolation and identification of chemical compounds from bark and have found polyphenol compounds.

Yellow birch (*Betula alleghaniensis* Britton) bark contains triterpene constituents such as lupeol, betulin, and betulilnic acid, which have high biological activity (Habiyaremye et al. 2002). Clearly, tree bark has potential as a natural source of antioxidants.

Chamaecyparis lawsoniana (A. Murray) Parl., also known as Port-Orford cedar, Oregon white-cedar, or Lawson cypress, is a large tree of 43–55 m in height and 1.2–1.8 m in diameter. Its distribution is restricted to the coastal forests of southwestern Oregon and northern California in the USA (Harlow et al. 1978). Recent research has shown that a great number of fragrant plants and trees, such as *Ch. lawsoniana*, contain chemical compounds exhibiting bioactivity, such as antioxidant and antimicrobial properties (Dapkevicius et al. 1998; Springfield et al. 2003; Miliauskas et al. 2004). Previous work has shown that *Ch. lawsoniana* wood has excellent decay and termite resistance (Morrell and Sexton 1987; McDaniel 1989; Tucker et al. 2000; Craig et al. 2004).

Besides the considerable bioactivity of *Ch. lawsoniana*, we have previously evaluated the antioxidant activity of the crude extract of the wood and bark of this species and found that the bark extract had higher potential antioxidant activity (Gao et al. 2006). Therefore, the objective of this study was to perform advanced isolation work on bark crude extract and to evaluate its activities. Several in vitro assays, including radical-scavenging assays with 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH•) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), were carried out to evaluate the antioxidant activity of extracts from *Ch. lawsoniana* bark. Previous research has shown that phenolic compounds extracted from medicinal plants are potential antioxidants (Yen et al. 1993). Hence, we quantitatively analyzed the total phenolic content in various fractions from bark extracts.

Materials and methods

Chemicals

Potassium peroxydisulfate, ABTS, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid, and ferrozine sodium salt

were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ferrous chloride, gallic acid, DPPH•, and EDTA dihydrate, disodium salt were purchased from Alfa Aesar (Ward Hill, MA, USA), while butylated hydroxyltoluene (BHT) was purchased from Alfa Aesar (Lancaster, UK). All other chemicals were of standard analytical grade, except for methanol used in the DPPH• assay, which was HPLC grade, and the ethanol used in the ABTS test, which was spectrophotometry grade.

Preparation of extracts

Ch. lawsoniana bark was 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. Extraction of 100 g of particles was conducted with methanol (1 l) in a Soxhlet extractor until the solvent became colorless. The extracts were concentrated, suspended in deionized water, and sequentially partitioned with *n*-hexane, ethyl acetate, and *n*-butanol to obtain four different fractions. Fractions were collected, dried under a rotary evaporator, lyophilized in a freeze-drier, and kept in the dark at 4°C until testing. Extract yield with *n*-hexane, ethyl acetate, *n*-butanol, and deionized water from *Ch. lawsoniana* were 5.077%, 7.739%, 2.421% and 0.6423% (w/w), respectively.

Evaluation of antioxidant activity

DPPH• radical scavenging assay 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 (Soler-Rivas et al. 2000; Koleva et al. 2002). This transformation results in a change in color from purple to yellow, which was 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) (Cuendet et al. 1997; Burits and Bucar 2000). The reaction mixture was vigorously shaken by hand and then kept in the dark for 30 min under ambient conditions. The absorbance was measured at 512 nm, and the antioxidant capacity was expressed as percentage inhibition, calculated using the following formula,

$$\text{Inhibition (\%)} = 100 \times (A_0 - A_1)/A_0, \quad (1)$$

where A_0 is the absorbance of the blank and A_1 the absorbance of the sample with extract at 512 nm.

IC_{50} is the antioxidant concentration that inhibits the DPPH• reaction by 50% under the experimental conditions. This was calculated by plotting percentage inhibition against the extract concentration. Low IC_{50} values indicate high radical-scavenging activity. In this experiment, a synthetic antioxidant reagent, butylated hyroxytoluene (BHT), was used as a positive control. All analyses were run in triplicate and averaged.

$ABTS^{+\cdot}$ radical cation scavenging The $ABTS^{+\cdot}$ scavenging test is widely used to determine the antioxidant activity of both hydrophilic and lipophilic compounds. The reaction between ABTS and potassium persulfate directly generates the blue/green $ABTS^{+\cdot}$ 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 as described by Re et al. (1999) with some modifications. $ABTS^{+\cdot}$ was generated by mixing 5 ml of 7 mM ABTS with 88 μl of 140 mM $K_2S_2O_8$ in the dark at room temperature (23°C) for 16 h. The solution was diluted with 50% ethanol to achieve an absorbance of 0.7 ± 0.05 at 734 nm. The radical-scavenging activity was assessed by mixing 5 ml of this $ABTS^{+\cdot}$ solution (absorbance 0.7 ± 0.05) with 0.1 ml of bark extracts or negative control (methanol). The final absorbance was measured at 734 nm. The percentage inhibition of $ABTS^{+\cdot}$ was calculated using Eq. (1); in this case A_0 is the absorbance of the blank and A_1 the absorbance with the extract or BHT at 734 nm. All experiments were performed in triplicate.

Determination of total phenolic content (TPC)

TPC of methanol crude extracts was determined according to the Folin-Ciocalteu method described by Singh et al. (2002), with slight modifications. Results are expressed as gallic acid equivalents (GAE), which reflect the phenolic content as the amount of gallic acid in mg per gram dry weight of the sample. Methanol solution of the bark extracts (0.5 ml) was mixed with a ten-fold dilution of Folin-Ciocalteu reagents (2.5 ml) and incubated for 2 min at room temperature before the addition of a sodium carbonate solution (2 ml, 7.5% w/v). The absorbance of the solution was measured at 765 nm after standing for 30 min at room temperature. Gallic acid solutions (0.5 ml) in the concentration range 0.2–0.025 mg ml^{-1} were used to prepare a calibration curve. This estimation of phenolic compounds in the extracts was carried out in triplicate, and the results were averaged.

Results

Effect of DPPH• scavenging activity

The radical-scavenging properties of the various extracts of *Ch. lawsoniana* are shown in Figure 1. All bark extracts of *Ch. lawsoniana* exhibited concentration-dependent DPPH• scavenging activity. Among the extracts isolated, fractions EA and n-Bu showed higher antioxidant activity in this assay system, with inhibition of 85.61% and 94.52%, respectively, which were significantly higher than the BHT positive control (67.41%) at a concentration of 29.41 μg ml^{-1} (Figure 1a). The deionized water fraction showed moderate activity, close to that of BHT, while the *n*-hexane fraction showed weaker activity compared to the other three fractions and the positive control. The IC_{50} values for *n*-hexane, ethyl acetate, *n*-butanol, and deionized water fractions were 115.4, 13.04, 6.53, and 22.48 μg ml^{-1}, respectively. The IC_{50} value of the reference compound, BHT, was approximately 19.27 μg ml^{-1}. The *n*-butanol fraction showed the lowest IC_{50}, which indicated it was the most effective against DPPH• compared with the well-known antioxidant BHT. The IC_{50} value of the *n*-hexane fraction was obtained by extrapolation of the data because of its low antioxidant activity.

Effect of $ABTS^{+\cdot}$ scavenging activity

An improvement of the method of Re et al. (1999) was applied in this study to obtain the radical-scavenging data shown in Figure 1b. The control, BHT, and the four extracts exhibited concentration-dependent $ABTS^{+\cdot}$ scavenging activity. At concentrations from 1.765 to 5.882 μg ml^{-1}, the gradients of the curves of percentage inhibition versus concentration for n-Bu and EA were steeper than for the other two extracts, indicating that in this concentration range the anti-radical activity increased rapidly with sample concentration. The gradi-

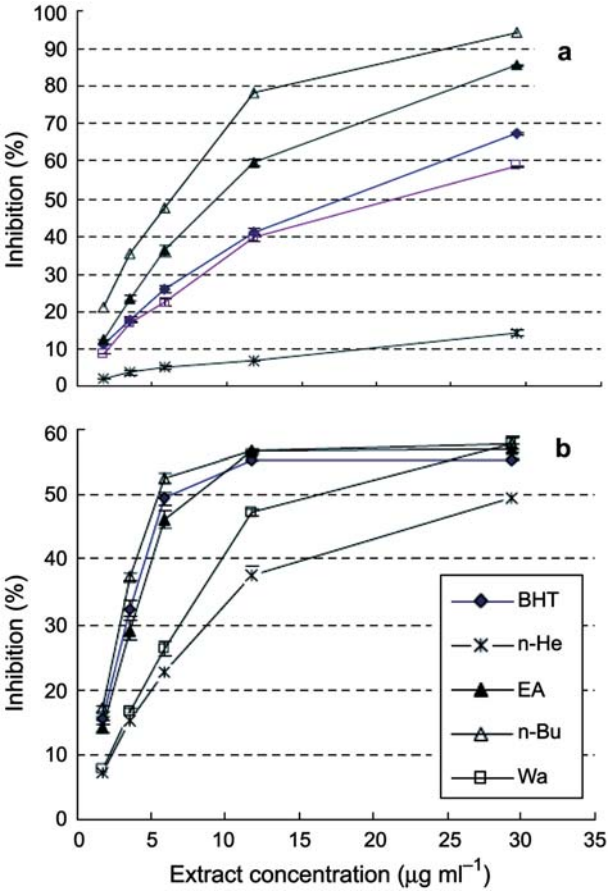

Figure 1 (a) DPPH• and (b) $ABTS^{+}$ scavenging capability of bark extracts of Port-Orford cedar. Note: n-He, EA, n-Bu and Wa indicate bark extracts fractionated in *n*-hexane, ethyl acetate, *n*-butanol, and deionized water, respectively. Each value is the mean ± SD of three measurements. Some of the SD values are too small to show.

ents increased slowly or remained constant at higher concentrations. In these instances, $ABTS^{+\bullet}$ may have been largely reduced and the color was not proportional to the amount of radical scavenger. Among the four extracts of *Ch. lawsoniana*, n-Bu showed higher inhibition of $ABTS^{+\bullet}$ at all test concentrations than the synthetic antioxidant, BHT, under the same experimental conditions, followed by EA and Wa fractions. The *n*-hexane fraction showed the weakest radical-scavenging ability.

TPC of *Ch. lawsoniana* extracts

The total phenolics content (mg g^{-1}) in methanol extracts was determined from a regression equation for the calibration curve ($y = 0.004663x + 0.0565$, $R^2 = 0.99$) and expressed in GAE. Table 1 lists the distribution of phenolic compounds in the four fractions from bark of *Ch. lawsoniana*. The *n*-butanol fraction contained the highest amount, 428.5.5 mg GAE g^{-1}, followed by ethyl acetate, deionized water, and *n*-hexane fractions. However, GAE g^{-1} dry bark powder for the EA fraction showed the highest value because it had the highest extract yield (7.739%, w/w) of the four samples. Therefore, ethyl acetate is a good solvent for separating antioxidant polyphenols. Figure 2 shows that TPC in the extracts

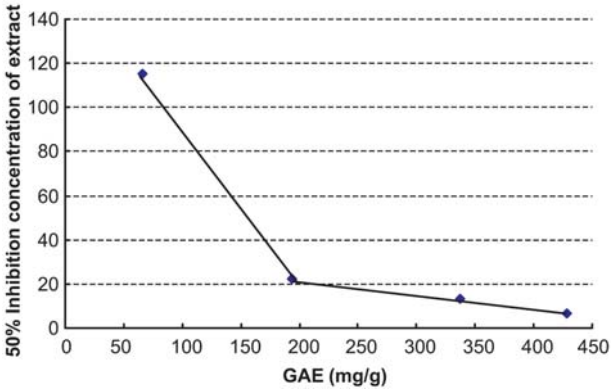

Figure 2 Relationship between total phenol content and IC_{50} values (DPPH•). Note: IC_{50} is concentration showing 50% inhibition and the total phenol content is expressed as gallic acid equivalents (GAE).

correlates well with the IC_{50} of the DPPH• scavenging assay. This correlation suggests that phenolic compounds are likely to contribute to the radical-scavenging activity of the methanol extracts.

Discussion

Natural phenolic products have long been investigated for their potential as commercial antioxidants. One major problem is that most antioxidants are used in plastics, and most plastics are hydrophobic. Thus, a hydrophobic antioxidant such as BHT is needed to prevent the antioxidant from negatively influencing the plastic surface. Another problem is the thermal stability of the natural phenolic antioxidant, with most plastics processed at quite high temperatures. Many of the natural antioxidants, such as condensed tannins from bark, have a brown color. On the other hand, simple phenolic compounds such as BHT are mostly white or colorless. As simple phenolics polymerize, the color changes from colorless to yellow or brown. We would not expect these materials to be much more stable than wood. We know enough about these extracts to expect some of the phenolic compounds to show some discoloration when heated to temperatures as high as 200°C.

Future work is advised to provide additional information about the chemical composition of the extracts. Further refinement is needed to establish the limitations of these antioxidants. Such natural antioxidants, if targeted for use in plastic packaging, would be most suitable in "natural" packaging, where color is not an issue. Many

Table 1 Total phenolic content of different extracts from the bark of Port-Orford cedar.

Sample	Total phenolic content (mg GAE g^{-1})	
	Dry extract	Dry bark powder
n-Hexane	65.7 (2.33)	3.336 (0.12)
Ethyl acetate	337.0 (1.43)	26.08 (0.11)
n-Butanol	428.5 (2.48)	10.38 (0.06)
Deionized water	193.8 (2.89)	1.245 (0.02)

GAE, gallic acid equivalents. Values in parentheses are the standard deviation.

plastics are colored (or applied to colored substrates, such as brown packaging paperboards), and any color from the antioxidant would likely be of little issue. While most of the antioxidants find their way into plastics, the rest find their way into other applications. A more suitable application for natural antioxidants appears to be for wood preservation. Since wood is hydrophilic, it is a good match for hydrophilic antioxidants. The combination of BHT with chlorothalonil gave enhanced efficacy in ground-contact field tests (Green and Schultz 2003).

Conclusions

Ch. lawsoniana bark can be used as an easily accessible source of natural antioxidants. Ethyl acetate has the highest extraction power for phenols, followed by *n*-BuOH. The antioxidant mechanisms of *Ch. lawsoniana* extracts may be due to the strong hydrogen-donating ability of the phenolic compounds contained in the bark, which can reduce the concentrations of DPPH• and $ABTS^{+\bullet}$ free radicals. The total phenolic content of the extracts correlates well with the radical-scavenging activity. Studies are in progress to isolate and identify the chemical compounds that contribute to the total anti-radical activities and to better understand their mechanism of action as antioxidants.

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