Research article

Antioxidant activity and low cytotoxicity of extracts and isolated compounds from *Araucaria angustifolia* dead bark

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The antioxidant activities of the extract and its relevant fraction as well as isolated compounds from the dead bark of *Araucaria angustifolia* are presented. This tree represents the Mixed Ombrophile Forest, which is endangered due to extensive logging. The dead bark of Araucaria is naturally discarded by the tree, and its hydroalcoholic crude extract has exhibited protective qualities against stress induced by H_2O_2 in cell culture. Using several *in vitro* models, here we describe the antioxidant potential of the crude extract and its component ethyl acetate fraction and also of some compounds isolated from the ethyl acetate fraction. We provide the first description of the isolation of two natural product afzelechin derivatives. The extract and isolated compounds, particularly *epi*afzelechin protocatechuate (**5**), displayed very high antioxidant activity, as did compound **4**, quercetin, which is well known for its antioxidant properties. In a DPPH assay, compound **5** exhibited an IC_{50} of 0.7 μM; in a lipid peroxidation assay; IC_{50} values of 21 μM and 35 μM were obtained when the oxidation was induced by UV and ascorbate free radicals, respectively.

Keywords: *Araucaria angustifolia*, bark, antioxidant, phenolics, *epi*afzelechin *p*-hydroxybenzoate, *epi*afzelechin protocatechuate, *epi*catechin

Introduction

The endemic tree *Araucaria angustifolia* (Brazilian pine) represents the Mixed Ombrophile Forest, which is one of the phytophysiognomies of the Atlantic Forest biome. Its timber has been widely exploited due to its high quality and, since 1992, this species has been considered as endangered. The Atlantic Forest law, which defines the rules and principles for its use and protection, incentivizes the sustainable exploitation of plants as a strategy to stimulate the recovery of forest remnants. The dead bark of *Araucaria* is naturally discarded by the tree and can be collected throughout the year without causing damage (Fig. 1). Its hydroalcoholic crude extract is rich in polyphenolic compounds.

The dead bark of *A. angustifolia* was investigated for the first time in this study, although previous studies have verified the antioxidant potential of leaf extracts.[1] Specifically, biflavonoids were isolated from the leaves of *A. angustifolia*,[2] while isoflavones,[3] lignans and nor-lignans,[4] and diterpenes[5] were isolated from the resin. The amentoflavone-type biflavonoids (C8″–C3′) have been shown to possess high antioxidant properties and a significant capacity to

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Figure 1 ***Araucaria angustifolia*, the arrow shows its dead bark**

quench singlet oxygen as well as to inhibit the oxidation of DNA and lipoperoxidation caused by free radicals.[1]

The objective of this work was to investigate the chemical composition of the dead bark of *A. angustifolia* and the antioxidant properties of this raw material, which can be collected without damage to the tree and has never been studied. The aim was to find molecules with potential application in health or cosmetics that may help efforts to preserve the plant. In this work, the dead bark of the endangered species *A. angustifolia* was extracted with ethanol-water (1:1) and partitioned with ethyl acetate; the acetate fraction (AF) was characterized. Several phenolic compounds were isolated and identified by spectroscopic techniques. These materials were explored for their antioxidant activity; in particular, their reactivity to DPPH was evaluated along with any ability to protect against lipid peroxidation induced by various reactive oxygen species (ROS). Additionally, the redox potential of each fraction and compound was determined.

Materials and methods

Plant material, extraction, and isolation

Dead bark of *A. angustifolia* was gathered gather from an experimental farm in General Carneiro (PR). The dead bark contained 17.68% humidity, which, after a natural drying period, reduced to 12.77% humidity. The crushed material (5–2 mm) was percolated with 50% ethanol in water at 70ºC and then centrifuged to give the hydroalcoholic liquid extract containing 5.25% dry residue. The extract was concentrated to furnish 10% dry residue. The concentrated crude hydroalcoholic extract (HE) was partitioned with ethyl acetate to obtain the acetate fraction (AF). The AF was subjected to column chromatography (CC) using silica gel and a gradient of dichloromethane–methanol (95:5 to 5:95) as eluent to yield the following compounds: benzoic acid (**1**), *p*-hydroxybenzoic acid (**2**), protocatechuic acid (**3**), and quercetin (**4**). The fractions were then subjected to further CC over the same system resulting in the isolation of two catechins: (–)-*epi*afzelechin protocatechuate (**5**) and (–)-*epi*afzelechin *p*-hydroxybenzoate (**6**). The late fractions of the previous separation were subjected to CC over cellulose using ethanol as eluent to give (–)-*epi*catechin (**7**). The structures of the isolated compounds **1–4** and **7** were elucidated by nuclear magnetic resonance analyses and compared to previously published data (Spectra Database SDBS for **1–3**, Sing and Chauhan[6] for compound **4**, and Foo *et al*.[7] for compound **7**). The spectroscopic data for compounds **5** and **6** are given below and Figure 2 summarises the structures of the compounds discussed.

(–)-(2R, 3R)-5,7-dihydroxy-2-(4-hydroxyphenyl) chroman-3-yl 3,4-dihydroxybenzoate, epi*afzelechin protocatechuate (**5**)*

Red gum, $[\alpha]_D$ = –36 (*c* = 0.35, EtOH); ^{1}H-NMR (acetone-d_6, 400 MHz): δ 7.53 (*d*,*J* = 1.9 Hz, H2″), 7.47 (*dd*, *J* = 8.2 and 1.9 Hz, H6′), 7.36 (*d*,*J* = 8.6 Hz, H2′,H6′), 6.90 (*d*,*J* = 8.2 Hz, H5″), 6.81 (*d*,*J* = 8.6 Hz, H3′, H5′), 6.03 (*d*,*J* = 2.4 Hz, H8), 5.93 (*d*,*J* = 2.4 Hz, H6), 4.90 (*bs*, H2), 4.17 (*bs*, H3), 2.88 (A of AB_q, *J* =16.3, 4.3, H4a), 2.74 (B of AB_q, *J* = 16.8, 2.5, H4b); ^{13}C-NMR (acetone-d_6, 100 MHz): δ 167.7 (CO), 160.1 (C4′), 157.6 (C5, C7), 157.2 (C9), 150.7 (C3″), 145.5 (C4″), 131.4 (C1′), 129.1 (C2′, C6′), 123.6 (C2″), 123.1 (C1″), 117.5 (C6″), 115.7 (C3′, C5′), 115.5 (C5″), 96.1 (C10), 95.7 (C6, C8), 79.5 (C2), 66.8 (C3), 29.4 (C4); ESI-MS *m*/*z* 409.1 $[M–H]^-$; ESI-MS/MS (daughter ions, 35%) *m*/*z* 365 $[M–CO_2]^-$ (10), 325 (83), 289 (100), 283, 273, 245, 205, 203, 153, 109 (found C, 65.59%; H, 4.05%. Calculated for $C_{22}H_{18}O_8$: C, 64.39%; H, 4.39%)

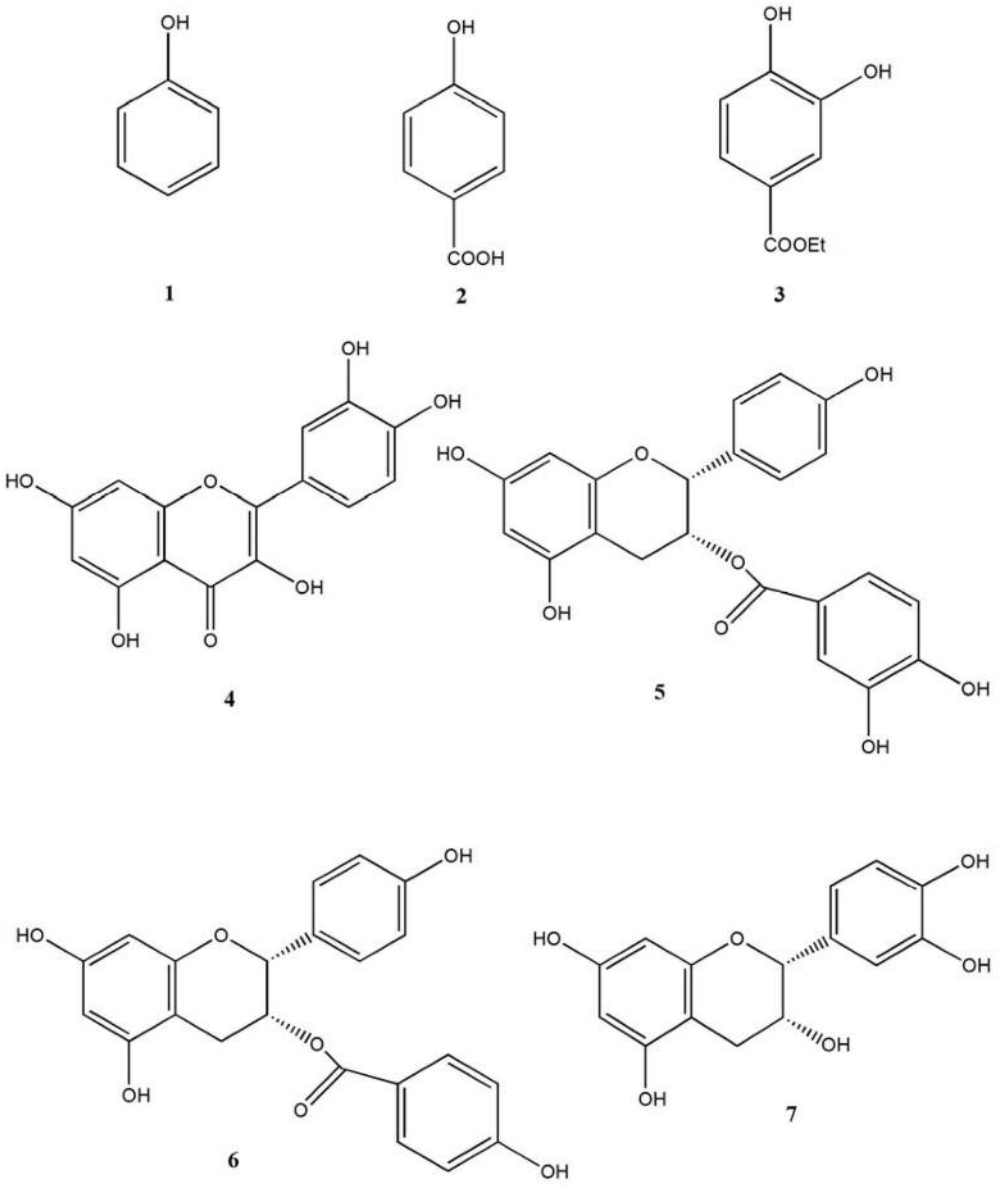

Figure 2 Compounds 1–7, isolated from *A. angustifolia* dead bark

(–)-(2R, 3R)-5,7-dihydroxy-2-(4-hydroxyphenyl) chroman-3-yl 4-hydroxybenzoate, epi*afzelechin* p-*hydroxybenzoate (**6**)*

Red gum, $[\alpha]_D$ = –56 (*c* = 0.39, EtOH); ^{1}H-NMR (MeOD, 400 MHz): δ7.85 (*d, J* = 8.7 Hz, H2″,H6″), 7.31 (*d, J* = 8.3 Hz, H2′,H6′), 6.86 (*d, J* = 8.6 Hz, H3″, H5″), 6.75 (*d, J* = 8.6 Hz, H3′, H5′), 5.99 (*d, J* = 2.3 Hz, H8), 5.88 (*d, J* = 2.3 Hz, H6), 4.88 (*bs*, H2), 4.17 (*bs*, H3), 2.86 (A of AB_q, *J* = 16.6, 4.5, H4a), 2.74 (B of AB_q, *J* = 16.8, 2.5, H4b); ^{13}C-NMR (acetone-d_6, 100 MHz): δ 162.5 (CO), 157.6 (C5, C7, C9), 157.2 (C4′), 132.7 (C4″), 131.5 (C1′), 129.1 (C2′, C6′, C2″, C6″), 122.9 (C1″), 115.9 (C3′, C5′), 115.5 (C3″, C5″), 99.7 (C10), 96.2 (C6), 95.7 (C8), 79.5 (C2), 66.8 (C3), 29.4 (C4); ESI-MS *m/z* 393.3 $[M–H]^-$; $C_{22}H_{18}O_7$: *m/z* 301 (80), 285 (60), 137 (100). Data in accordance with WO/2006/017981.

Spectrophotometric determination of phenolic compounds

The total phenolic content of *A. angustifolia* dead-bark extract was determined by the Folin–Ciocalteu method.[8] Total phenolics were expressed as milligram gallic acid equivalents per gram of extract or acetate fraction (AF). The total anthocyanin content was determined using the pH differential method described in Lapornik *et al.*[9] and expressed as milligram malvidin glucoside equivalents per gram of

extract or acetate fraction; total proanthocyanidin (condensed tannins) were determined using the vanillin assay.[10] The total amount of procyanidin was expressed as procyanidin B_2 equivalents per gram of extract or acetate fraction. All samples were analyzed in triplicate.

Liquid chromatography analysis

The HE was analyzed in an LC system consisting of a Waters 600 pump with a 2996 PDA detector, an automatic 717 plus injector, an in-line degasser AF, and Millennium Empower software. The injection mixtures (20 μl) were resolved through a Phenomenex Synergi Fusion RP 80Å column (250 × 4.6 mm). The mobile phase consisted of a linear gradient from 10–50% ultrapure water, acidified to 0.01% with H_3PO_4, over 25 min followed by 5 min to re-equilibrate the column to its initial condition. The flow rate was 0.8 ml/min. The separation was monitored at 280 nm, and the column oven was set to 35ºC. All solvents were LC grade and were degassed by an ultrasonic bath. The water was purified using a Milli-Q system. All solutions were filtered through 0.45-μm membranes. Samples were diluted 1:10 with the mobile phase and analyzed in triplicate.

Inducing oxidative stress in mouse L929 fibroblasts

The cytotoxicity/protection profile of the HE was evaluated in confluent L929 fibroblasts (BCRJ) propagated and maintained in Dulbecco's Modified Eagle's Medium (DMEM, glutamine 2 mM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and PSN (0.05 mg/ml penicillin, 0.05 mg/ml streptomycin, and 0.01 mg/ml gentamicin) at 37ºC in a 5% CO_2 humidified atmosphere. L929 fibroblasts (1×10^4 cells/ml, 100 μl per well) were seeded in a 96-well plate and, 24 h after plating, were treated with serial dilutions of the sample (100 μl per well, from 0.01 to 1000 μg/ml) in triplicate experiments. Twenty-four hours later, medium (with and without sample) was removed, and oxidative stress was induced by exposing fibroblasts to H_2O_2 at different concentrations (from 0.5 to 100 μM in DMEM/FBS 5%, 100 μl per well) over 90 min. Cells were allowed to recover in fresh medium for 48 h after being subjected to stress then analyzed for cell viability. Cell controls with or without H_2O_2 were employed, and acetyl cysteine was used as a positive antioxidant control. The results obtained using the cell line are expressed as the mean ± SEM and were analyzed by one-way analysis of variance (ANOVA). When significant values were found ($P < 0.05$), post hoc comparisons of mean values were carried out using Duncan's test.

Assaying oxidative stress using 1,1-diphenyl-2-picrylhydrazyl (DPPH)

The radical-scavenging activity of the extracts was measured by a method slightly modified from that reported by Vivot *et al*.[11] The assay involves incubating the reaction medium for 30 min at 37ºC in an ethanolic solution of 200 μM DPPH and measuring the optical density at 517 nm. The antioxidant activities of the HE, AF and compounds are expressed as the IC_{50}, defined as the concentration of the extract or compound required to reduce 50% of the DPPH free radicals, and calculated from a Hill Plot (GraphPad Prism v4).

Determination of the redox potential of the compounds

The oxidation potential of electrochemically active substances gives an estimate of their capacity to donate e^- to reduce other species. Cyclic voltammetry assays were performed on an Autolab PGSTAT30 potentiostat/galvanostat coupled to a computer with GPES v4.9 software. The rate scan was 100 mV/s, and the assays were conducted in a phosphate buffer solution (0.1 M, pH 6). The samples were tested immediately after preparation, at a concentration of 30 μg/ml, for HE or AF; the isolated compounds were tested at a concentration of 30 μM. The measurements were performed using a system of three electrodes a carbon paste work electrode, a platinum auxiliary electrode, and a reference electrode of Ag/AgCl. Quercetin was used as a positive control at the same concentration range of the tested samples.

Lipoperoxidation induced by UV light

The protection presented by the compounds against lipid peroxidation (LPO) induced by UV radiation was evaluated in soybean phosphatidylcholine (PC) liposomes and in rat microsomes. The PC liposomes were prepared by the method of lipid film hydration. Reaction medium containing the liposomes (25 mg/ml), with or without sample, was exposed for 60 min to UV radiation from a germicidal lamp (λ = 254 nm) positioned 10 cm from the sample. The extent of LPO was determined by the TBARS method.[12] Microsomes were prepared from rat liver by differential centrifugation with calcium aggregation,[13] and fractions were stored at –84ºC. The protein concentration was determined following the Lowry method.

The results are expressed as a percentage of the control, which was arbitrarily assigned the value of 100% peroxidation. The IC_{50} was calculated from the Hill Plot (GraphPad Prism v4).

Lipoperoxidation induced by ascorbyl radical

Lipid peroxidation was induced by the reaction system Fe/ascorbate, which generates the ascorbyl radical ($Asc^{\bullet-}$). To the reaction medium containing 25 mg/ml liposomes in 0.1 M Tris-HCl (pH 7.4), $FeSO_4$ (25 μM) and ascorbate (500 μM) were added. The samples were incubated for 30 min at 37°C. Incubations were performed with and without (control) different concentrations of the compounds. The extent of lipid peroxidation was determined by the TBARS method.[12] The results are expressed as a percentage of the control considering 100% peroxidation for the control. The IC_{50} was calculated from the Hill Plot (GraphPad Prism v4).

Lipoperoxidation induced by hydroxyl radical

The hydroxyl radical was produced in a Fenton reaction system H_2O_2/Fe^{3+}/ascorbate, which was adapted from Halliwell *et al*.[14] PC soybean liposomes (25 mg/ml) were added to reaction medium containing 20 μM $FeCl_3$, 2.8 mM H_2O_2, and 100 μM ascorbic acid. The samples were incubated for 30 min at 37°C. Incubations were performed with and without (control) different concentrations of the compounds. The extent of LPO was determined by the TBARS method.[12] The results are expressed as a percentage of the control considering 100% peroxidation for the control. The IC_{50} was calculated from the Hill Plot (GraphPad Prism v4).

Table 1 LC data of the hydroalcoholic extract (HE) of *A. angustifolia*

Retention time (min)	Wavelength (nm)	Derivative
8.44	259, 293	Unknown
12.52	252	Unknown
14.47	277	Flavanol
17.72	274	Flavanol
23.43	262, 325	Flavonol
23.89	265, 343	Flavonol
24.03	266, 343	Flavonol
25.99	278	Flavanol
33.63	274	Flavanol
35.33	369	Unknown
36.32	272	Flavanol
39.49	272	Flavanol

Octanol/water partition coefficient (log P)

The log P values were calculated for the isolated compounds using the software ACD/ChemSketch (Freeware).

Statistical analysis

The results were evaluated by the statistical software Prism v4, with the mean ± SD of experiments performed in triplicate.

Results

The spectrophotometric analysis of total phenolic content uncovered mean values of 64 mg/g and 33 mg/g of gallic acid equivalents for HE and AF,

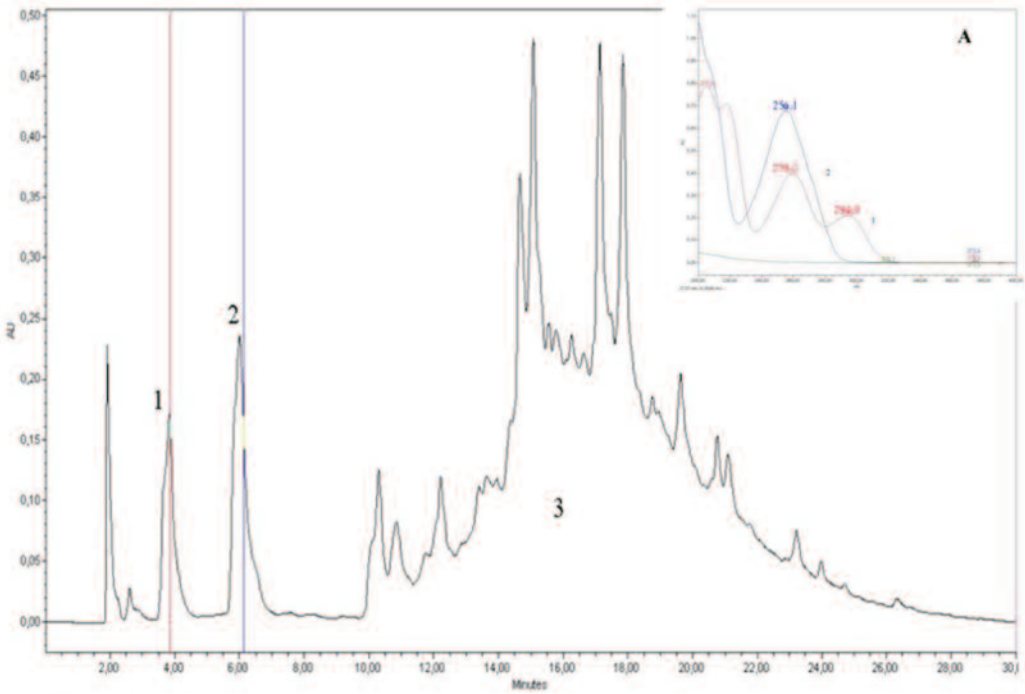

Figure 3 Chromatogram of hydroalcoholic extract (HE) of *A. angustifolia*. Detection: 280 nm. Peaks 1 and 2: unknown phenolics, peak 3: polymeric phenolics. Frame A: UV profile of compounds 1 and 2

Table 2 Reactivity with DPPH and redox potential of *A. angustifolia* dead-bark extract, fraction and isolated compounds

Compounds	DPPH reactivity (IC_{50})		Cyclic voltammetry	
	μg/ml	μM	Oxidation potential (mV)	Reduction potential (mV)
HE	1 ± 0.1	–	286	186
AF	0.9 ± 0.15	–	286	189
1	–	> 50	391	
2	–	10 ± 3	358	436
3	–	1 ± 0.05	522	69
4	–	0.6 ± 0.09	229	196
5	–	0.7 ± 0.05	284	182
6	–	11 ± 3	339	–
Quercetin*	–	0.6 ± 0.05	230	197

*Quercetin was used as positive control.

respectively. The total anthocyanin content was found to be 1.85 mg/g and 3.6 mg/g of malvidin glucoside equivalents, and the total proanthocyanidin content was measured at 12 mg/g and 5 mg/g of procyanidin B_2 equivalents for HE and AF, respectively.

The chromatographic profile of HE exhibited a high concentration of flavanols in the phenolic composition of the extract (Fig. 3 and Table 1). It addition, the presence of polymeric proanthocyanidins was observed (peak 3 in Fig. 3).

Isolated compounds **1**–**4** are widely found in plants, especially quercetin (**4**), which is well known for its antioxidant properties.[30] They are extensively oxidized and are probable degradation products of catechins **5** and **6**, which are described here for the first time as natural products. The structure of compound **5** is novel, while compound **6** was previously synthesized and tested as a proteasome inhibitor.[15]

To evaluate any cytotoxicity of the HE, it was incubated alone with L929 cells and examined by optical microscopy or in the MTT viability assay. Verification of cell viability was the first important step to determine the concentration of H_2O_2 to be used in the oxidative stress test.

The IC_{50} of H_2O_2 alone for L929 cells was 0.125 μM ($r = 0.98$; $P < 0.0001$). Pre-incubating L929 cells with 1 μg/ml of HE increased the IC_{50} to 0.44 μM ($r = 0.92$; $P < 0.01$), which suggests increased cellular protection against oxidative stress caused by H_2O_2. Increasing the HE concentration 1000-fold (1 mg/ml) boosted the degree of cellular protection by 131% in a dose-responsive manner. Only at high concentrations (1 mg/ml or 6 mM) did the acetyl cysteine control result in cellular protection. At lower concentrations (from 0.006 to 0.6 mM), no antioxidant activity was observed.

The DPPH assay evaluates the ability of molecules with antioxidant properties to scavenge free radicals. The results presented in Table 2 show that all substances tested, including the crude HE and AF, exhibit antioxidant potential; the data indicate that, after the known compound quercetin (**4**), catechin **5** is the most potent of the isolated compounds in preventing the oxidation of DPPH. The isolated compounds exhibited antioxidant activity in the following order: **4** > **5** > **3** > **2** > **6** > **1**, and the ethyl acetate fraction was little more active then the crude extract (AF > HE). The oxidation potentials obtained by cyclic voltammetry for all samples were in accordance with the reactivity with DPPH, except for compound **3**. It has to be considered that the DPPH

Table 3 Prevention of liposome lipid peroxidation induced by UV light and by ascorbyl and hydroxyl radicals of *A. angustifolia* dead-bark extract, fraction and isolated compounds

Compounds	Lipid peroxidation (IC_{50})						Log P
	UV radiation		Ascorbyl		Hydroxyl		
	μg/ml	μM	μg/ml	μM	μg/ml	μM	
HE	36 ± 3	–	18 ± 1	–	12 ± 1.5	–	
AF	25 ± 2.5	–	17 ± 1	–	22 ± 2	–	
1	–	> 50	–	> 50	–	> 50	1.89 ± 0.21
2	–	> 50	–	> 50	–	> 50	1.42 ± 0.22
3	–	> 50	–	> 50	–	> 50	1.16 ± 0.24
4	–	9 ± 1	–	30 ± 2	–	> 50	3.06 ± 0.2
5	–	21 ± 2	–	35 ± 5	–	> 50	3.43 ± 0.21
6	–	> 50	–	> 50	–	> 50	3.6 ± 0.39
Quercetin*	–	10 ± 0.5	–	28 ± 1	–	> 50	3.06 ± 0.2

*Quercetin (Sigma) used as positive control

Table 4 Prevention of microsome lipid peroxidation induced by UV light and ascorbyl radical of compound 5

Compound	Lipid peroxidation (IC_{50})	
	UV radiation μM	Ascorbyl μM
5	18 ± 4	6 ± 0.25

assay was carried out in ethanolic medium and the cyclic voltammetry was performed in aqueous medium. In aqueous medium, the e^- transfer may be inhibited in some cases.

The observed reactivity of the compounds with DPPH is in accordance with the redox potential measured in the electrochemical assay. The ideal conditions for the electrochemical assay were obtained from prior screening of pH, rate scanning, sample concentrations, and cyclic voltammetry.

The substances were also evaluated as antagonists of UV-induced lipid peroxidation in phosphatidylcholine (PC) liposomes. The assay was carried out to optimize the concentrations of each substance. After the membranes were exposed to UV light, a significant increase in TBARS formation was observed. Two controls were run in parallel: (i) without the oxidation source; and (ii) without the sample in order to evaluate the basal peroxidation of the liposomes. Table 3 presents the IC_{50} values for each substance and reveals that all substances tested, including HE and AF, were able to prevent UV-induced lipid peroxidation at relatively low concentrations; catechin **5** was the most potent among the isolated compounds after compound **4**.

Lipoperoxidation was also evaluated using liver microsomes as an ROS target. Two different sources of membrane oxidizers were used: UV light and ascorbyl radicals. However, the assays were conducted only with catechin **5** because it showed the best results with liposomes, after compound **4**. Compound **5** was also able to protect microsomal membranes against ROS at relatively low concentrations; IC_{50} values were measured at 6 ± 0.25 μM and 18 ± 4 μM for ascorbyl radicals and UV radiation, respectively (Table 4).

Discussion

In general, high phenol content correlates significantly with high antioxidant capability. Catechins and proanthocyanidins are phenols that protect against non-specific biological oxidation and possess proven activity for tumor prevention[16] and photoprotection.[17] Green tea catechins have been widely studied and have demonstrated, among other benefits, a collagen stabilizing effect as well as diminution of histamine liberation (anti-allergic effect).[18] Compounds **5** and **6** are esterified derivatives of *epi*afzelechin (*cis*-3,4′,5,7-tetrahydroxyflavane), which has been isolated from other sources and has exhibited antimicrobial,[19] cyclooxygenase-1 inhibitory, and anti-inflammatory effects.[20] Oligomers of *epi*afzelechin act through various modes of action to suppress herpes simplex virus propagation,[21] and polymers of *epi*afzelechin have been shown to inhibit HIV-1 protease.[22]

Encouraged by the lack of cytotoxicity of HE from dead bark of *A. angustifolia* together with its protective qualities against H_2O_2-induced oxidative stress, the antioxidant activity of the ethyl acetate fraction (AF), and its derivatives were evaluated by various methods. Although all samples showed high concentration-dependent reactivity for free radicals, the HE and AF samples were also potent. The high scavenging activity may result from a synergy among several substances present in HE and AF such as phenolic compounds, amino acids, pigments, and others.[23]

According to Saija *et al.*,[24] the LogP and a number of structural features are determinants of the scavenging activity or antioxidant potential of flavonoids: (i) the *o*-dihydroxy (catechol) of the B ring; (ii) the double bond 2–3 in conjugation with the 4-oxo functional group; and (iii) the presence of hydroxyl groups at positions 3, 5, and 7. Quercetin (**4**) possesses these three prerequisites, while compounds **5** and **6** have only one hydroxyl group at position 4' of the B ring; the hydroxyl group at position 3 was esterified to the 3,4-dihydroxybenzoic acid moiety in compound **5**, which is most likely responsible for its higher antioxidant activity because position 3 of compound **6** is esterified to *p*-hydroxy-benzoic acid (**4** > **5** > **3** > **2** > **6** > **1** and AF > HE).

Taking into account that the electrochemical behavior of these substances depends on their structural characteristics (viz. the number and positions of phenolic hydroxyls), cyclic voltammetry assays were performed. The voltammograms obtained for the majority of compounds correspond with the results of the DPPH assay (Table 2). The oxidation potential of a substance give an estimate of the requisite energy to donate one electron; therefore, substances with relatively low oxidation potentials donate an electron more easily and consequently exhibit higher antioxidant activity.[25] Given that quercetin (**4**) isolated from the plant studied here and quercetin commercially obtained, used as a positive control in the DPPH assay, exhibits an oxidation peak at 229 mV, while catechin **5** exhibits an oxidation peak at 284 mV, **6** at 339 mV, and **3** at 532 mV, it is possible that the number of hydroxyl groups could be responsible for these peaks.

Evaluating the results presented in Table 2, compounds **6-2** and **5-3**, which are structurally related, have similar antioxidant profiles with respect to both the DPPH and cyclic voltammetry assays, which suggests that meta phenolic hydroxyls lead to greater activity of the compounds. The discrepancy between the results of compound **3** in the DPPH assay and voltammetry is probably related to the interference of the reaction medium of each assay. DPPH was carried out in ethanolic medium and the voltammetry was performed in aqueous medium. The high oxidation potential obtained for the compound **3** (523 mV) and the high reactivity with DPPH (IC_{50} = 1 μM) was probably due to the difficulties in e^- transfer in aqueous medium, due to the ionization grade of the carboxyl under the ring interfering with the hydrogen donation from the *ortho* and *para* positions, a phenomenon that apparently does not occur in ethanolic medium.[30]

Lipid peroxidation describes ROS-mediated oxidation of polyunsaturated fatty acids present in cell membranes and lipoproteins. There is a relationship between lipid composition and the physical state of cell membranes and the conformation and activity of enzymes and receptors. Lipids that constitute membranes can be chemically modified by reactive species, resulting in alteration of their functions and features. Liposomes, because they have similar characteristics to biological membranes but are more easily manipulated, are an accepted *in vitro* model for studying peroxidation.

All substances were evaluated for their ability to protect against lipid bilayer peroxidation (Table 3). In this case, lipid peroxidation was induced by UV light and by hydroxyl and ascorbyl free radicals. Exposure to UV light produced ROS and consequent oxidative stress.[26] Among the ROS, the hydroxyl radical produced by the Fenton reaction is extremely reactive. In addition, the ascorbyl radical is generated by the Fenton reaction when accompanied by Cu^{2+} or Fe(III).[27] The ascorbyl radical presents a longer half-life compared to most ROS and is used as an oxidative stress biomarker in plasma in the form of the ascorbyl/ascorbate ratio.[28]

Aside from the structural features that bestow flavonoids with free radical scavenging activity, flavonoids also act as an anchor through their chemical bonds to the polar head-groups of phospholipids thus forming a reversible chemical complex. It has been suggested that, depending on solubility, there is a relationship between a flavonoid's interaction with a model membrane and formation of a flavonoid–phospholipid complex.[24]

All substances protected the liposomal membrane in a concentration-dependent fashion; increasing their concentrations caused the TBARS value to decrease. The most protective substances were HE, FA, and compounds **4** and **5**.

There is evidence linking UV-induced reactive oxygen species, including hydroxyl radicals, to the degradation of phospholipids.[26] Additionally, Mandal and Chatterjee[29] showed that liposomal membrane leakage is directly related to UV light or sunlight exposure. They also showed that an increase in radiation concomitantly increased membrane leakage and lipoperoxidation. The results presented in Table 3 show that, among the isolated substances, besides quercetin (**4**), compound **5** was more effective in protecting membranes against UV radiation damage (IC_{50} of 21 μM), while the IC_{50} values obtained for the majority of the compounds were higher than 50 μM. The IC_{50} for AF was 25 μg/ml, suggesting interactions among the several components of the fraction.

From the microsomal lipoperoxidation assays, one can see that most substances yielded similar results when membrane damage was induced by UV light, although it is important to note the elevated activity of HE and AF. The protective ability of catechin **5** against microsomal peroxidation was evaluated in the context of the ascorbyl radical and UV radiation and exhibited IC_{50} values of 6 ± 0.25 μM and 18 ± 4 μM, respectively.

These results validate the high protective activity observed for extracts from dead bark of *A. angustifolia* against lipid peroxidation in liver microsomes of rats, a natural membrane model, that have different compositions of proteins, metals, intrinsic antioxidants, and other components when compared with liposomes.

Given that particular structural features did not always correlate directly with the scavenging potential of the compounds studied herein, we suggest that the hydrophobicity (LogP) of the compounds should be analyzed for its contribution to functionality. It is known that flavonoids form reversible physico-chemical complexes via alignment of the polar head-groups of most phospholipids; as such, may be a relationship between the solubility of flavonoids and their interactions with model membranes.[24] According to Rice-Evans,[30] a flavonoid's partition coefficient is one of the parameters that define the antioxidant activity in the lipophilic phase. The LogP values (Table 3) are consistent with most results obtained from the lipid peroxidation protection assays; for example, quercetin (**4**) and catechin **5** possess high partition coefficients (3.06 and 3.43, respectively) and were the most active among the isolated compounds. The lipophilicity of compounds **1**, **2**, **3**, **6** may prejudice their activity as lipid membranes protectors. Previous

work reported similar behavior, that is, for each biological activity there is an appropriate range of hydrophobicity (LogP).[24,31,32]

Conclusions

Compounds **4** and **5** have the combination of requisites to be good antioxidants in hydrophilic and hydrophobic phases, namely: (i) the facility to donate e^-; and (ii) appropriate oxidation potential and lipophilicity. Similar considerations may apply to the fractions AF and HE, since they partially accord with these requisites, according to their composition. With these practical results in hand, we can estimate the potential of *A. angustifolia* dead bark as an active novel raw material for pharmaceutical and/or cosmetic products, paying special attention to the preservation of forest lands and sustainability of this novel source.

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