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Full Length Research Paper

Free radical scavenging activity of *Pterogyne nitens* Tul. (Fabaceae)

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As part of our ongoing research on antioxidant agents from Brazilian flora, twenty extracts and fractions obtained from *Pterogyne nitens* Tulasne (Fabaceae) were screened for free radical scavenging activity by using ABTS [2,2'-azinobis(3-ethylenebenzothiazoline-6-sulfonic acid)] and DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) radicals colorimetric assay and β-carotene bleaching test. The strongest activity was found in ethyl acetate fraction from the stem barks, exhibiting IC_{50} values (in µg/ml) of 2.10 ± 0.1 and 10.2 ± 0.3 on $ABTS^{\cdot+}$ and $DPPH^{\cdot}$, respectively. Additionally, chromatographic fractionation of stem barks yielding myricetin, quercitrin and mirycetrin, three flavonols with remarkable antioxidant activity.

Key words: Antioxidant, free radical scavenging activity, DPPH, ABTS, *Pterogyne nitens*, Leguminosae, Fabaceae, Caesalpinioideae, flavonoid, flavonol.

INTRODUCTION

Discovery and developing of new pharmacologically active compounds from natural sources is not a new phenomenon. Natural products of different structural patterns have proven active against free radicals (Cai et al., 2006) and screening of extracts is a valid strategy being exploited to discover antioxidant compounds isolated of microorganisms and plants (Banerjee et al., 2008; Kumar et al., 2008; Ruiz-Téran et al., 2008; Zheng et al., 2008).

In this context, there has been renewed alert in the rapid rate of plant species extinction, which reduces the time left to explore the remaining resources of natural compounds (Pinto et al., 2002). Urgent efforts are thus mandatory to collect and screen plants in order to determine if they should be conserved for future beneficial use of humankind (Baker et al., 1995).

Among legumes of the Caesalpinioideae subfamily, *Pterogyne nitens* Tulasne (Fabaceae) popularly named "bálsamo", "cocal", "amendoim-bravo", "yvi-raró" is the sole member of the genus, and has been submitted to a strong anthropogenic impact, which have led to its numerical population retraction, and classification into the category of species in critical risk. It is found only in small non-protected areas, and is therefore, subjected to the prompt possibility of extinction (Carvalho, 1994).

P. nitens is a beautiful legume tree, and the sole member of the genus, which is distributed mainly in South America and Tropical East Africa (Burkart, 1952; Lorenzi, 1998). Ethnopharmacological data of this species has not been frequently reported, and cold aqueous preparations from stem barks, have been used by Paraguayan natives in the treating of parasitic diseases, mainly of ascariasis (Crivos et al., 2007). Our previous phytochemical studies on *P. nitens* have resulted in the presence of guanidine alkaloids, which exhibited cytotoxic activity against to Chinese hamster ovary cells (Bolzani et al., 1995) and flavonoids with myeloperoxidase inhibitory activity (Regasini et al., 2008a,b; Fernandes et al., 2008).

Thus, the aim of the current study was to screen the extracts and fractions of branches, stem barks, green

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fruits, roots and compounds from *P. nitens* for a potential antioxidant activity on ABTS [2,2'-azinobis(3-ethylenebenzothiazoline-6-sulfonic acid)] and DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) radicals, as well as bleaching experiment on β-carotene.

MATERIALS AND METHODS

Plant material

Roots, branches, green fruits and stem barks of *P. nitens* were collected in the Botanic Garden of São Paulo by Ph.D. Maria C. M. Young, São Paulo State, Brazil, in January 2005 and identified by Ph.D. Inês Cordeiro (IBt-SMA). A voucher specimen (SP204319-b) has been deposited in the herbarium "Maria E. P. Kauffman" of the Botanic Institute (São Paulo, SP, Brazil).

Extraction

Shade-dried, powdered plant material was first defatted with hexane and exhaustively extracted by re-maceration with ethanol. The solvent was evaporated at low temperature under reduced pressure to yield thick syrup which was dispersed in methanol-water (4:1) and then successively partitioned with ethyl acetate and *n*-butanol. Samples from hexane and ethanol extracts, ethyl acetate, *n*-butanol, as well as lyophilized hydromethanol fractions, were further used in the potential antioxidant tests.

Isolation and identification of flavonols 1 – 3

The ethyl acetate fraction of the stem barks (320 mg) was subjected to preparative Gel Permeation Chromatography (GPC) on a Sephadex LH-20 (Pharmacia®) column (185 x 5.0 cm i.d.) and eluted with methanol. Twenty five fractions (50.0 ml) were collected and checked by TLC on silica gel F254 plates (Merck®) eluted with a mixture of ethyl acetate/water/formic acid/acetic acid (100:27:11:11). Fractions 12 - 16 (120 mg) were purified by repeated column chromatography (CC) with silica gel (Merck®) eluted with ethyl acetate:methanol (7:3), furnishing myricetin (1; 21 mg), quercitrin (2; 13 mg) and myricetrin (3, 7.0 mg). The molecular structures of these flavonoids were identified by comparison with literature data, mainly ^{1}H and ^{13}C NMR δ values (Markham et al., 1978).

Bleaching experiment on β-carotene

The qualitative test was carried out according to the method of Ruiz-Terán et al. (2008) with modifications, where 2.5 mg of a dried samples were diluted with 1 ml of dissolvent, then an aliquot (25 µl) of each sample was loaded onto the baseline of the TLC plate (Merck®). After developing and drying, plates were sprayed out with a 0.05 % solution of β-carotene in dichloromethane and then the TLC plates were exposed under a UV light (365 nm) for 1.5 h. A positive antioxidant reaction was considered to be present when yellow spots appeared on a white background.

Free radical scavenging activity (FRSA)

Various concentrations of the samples (extracts, fractions and flavonols **1-3**) were assayed and their FRSA against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ were evaluated. The results were expressed by using mean values obtained from triplicates as percentage of radical reduced (inhibition %) calculated from the equation: Inhibition % = [1–(A_{sample}/A) X 100], where A is test absorbance without sample (only ethanol and free radical) and A_{sample} is test absorbance with samples. Concentration providing 50% inhibition (IC_{50}) was calculated form the graph plotted inhibition percentage against concentration tested. Synthetic antioxidant Trolox® was adopted as positive control.

DPPH is a stable free radical that, when dissolved in ethanol, has purple color. Loss of this colorization indicates FRSA. Ethanol solutions of samples at various concentrations (1 - 100 µg/ml) were individually added to 0.6 µM DPPH in ethanol. The reaction (total volume 1.0 ml) was shaken vigorously and allowed to react at 25°C. After 15 min, remaining $DPPH^{\bullet}$ was determined colorimetrically at 531 nm, using absolute ethanol as a blank (Soares et al., 1997).

$ABTS^{\bullet+}$ was prepared by reacting 5 ml of 7 mM ABTS aqueous solution with 88 µl of 140 mM potassium persulphate (molar ratio 1:0.35) and the mixture allowed to stand in the dark at room temperature for 12 - 16 h before use (Pellegrini et al., 1999). Prior to assay this $ABTS^{\bullet+}$ stock solution was diluted with KH_2PO_4/K_2HPO_4 (100 mM, pH 7.0, diluted 1:10 before use) buffer solution (ratio 1:88) to give an absorbance at 734 nm of 0.414 ± 0.013 (n = 40). 1 ml $ABTS^{\bullet+}$ was then added to glass test tubes containing various concentrations (0.1 - 100 µg/ml) of each sample and mixed for 15 s. Tubes were incubated for 30 min and then read at 734 nm. A lower absorbance of the reaction mixture indicated higher FRSA.

RESULTS AND DISCUSSION

Human cells are constantly exposed to free radicals and reactive oxygen/nitrogen species (RNOS) generated by a number of biotic and abiotic factors such as irradiation, atmospheric and food pollutants or byproducts of metabolic processes. When the exposure overwhelms endogenous preventive systems, cells are exposed to a potentially harmful load of oxidants, leading to various free-radical-induced noxious effects (Ferguson et al., 2004).

Thus, free radicals are implicated in the pathogenesis of various human diseases such as myocardial and cerebral ischemia, arteriosclerosis, diabetes, rheumatoid arthritis, inflammation, cancer-initiation and aging processes (Coyle and Puttfarcken, 1993). Therefore, there is growing interest in free radical scavengers having the potential as protective agents against these diseases (Oluwaseun and Ganiyu, 2008; Zhu et al., 2008).

In the present work, two extracts (hexane and ethanol) and three fractions (ethyl acetate, *n*-butanol and hydromethanol) of the branches, stem barks, green fruits and roots of *P. nitens* were evaluated for free radical scavenging activity (FRSA) and the results are shown in Table 1.

In general, ethanol extracts from stem barks and green fruits exhibited stronger FRSA than did those from roots and branches against $ABTS^{\bullet+}$. The low-polarity extracts obtained by extraction with hexane proved to be not active on two tested free radicals, since the concentration at which this extracts showed activity was over 100 µg/ml. Hydromethanol and *n*-butanol fractions were less effective than ethyl acetate fractions, suggesting that the potential anti-$DPPH^{\bullet}$ and anti-$ABTS^{\bullet+}$ compounds were in

Table 1. Results of scavenger activity of *P. nitens* on ABTS and DPPH radicals expressed by IC_{50} ($\mu g.mL^{-1}$), and bleaching experiment on β-carotene.

Plant part or compounds	Extracts or fractions tested	β-carotene[a,b,c]	$ABTS^{\bullet+}$	$DPPH^{\bullet}$
Roots	Hexane	–	> 100	> 100
	Ethanol	+	19.0 ± 0.8	> 100
	Ethyl Acetate	++	10.5 ± 0.7	> 100
	n-Butanol	+	18.5 ± 0.7	> 100
	Hydromethanol	–	> 100	> 100
Green fruits	Hexane	–	> 100	> 100
	Ethanol	++	8.55 ± 0.7	> 100
	Ethyl Acetate	+++	4.53 ± 0.7	81.0 ± 5.4
	n-Butanol	+	21.0 ± 0.6	> 100
	Hydromethanol	–	> 100	> 100
Branches	Hexane	–	> 100	> 100
	Ethanol	++	39.0 ± 1.8	> 100
	Ethyl Acetate	++	33.0 ± 2.0	9.53 ± 0.7
	n-Butanol	++	38.5 ± 2.4	71.0 ± 3.9
	Hydromethanol	–	> 100	> 100
Stem barks	Hexane	+	> 100	> 100
	Ethanol	+++	4.22 ± 0.2	21.0 ± 1.1
	Ethyl Acetate	+++	2.10 ± 0.1	10.2 ± 0.3
	n-Butanol	+++	14.5 ± 0.2	4.10 ± 0.3
	Hydromethanol	–	> 100	> 100
Myricetin (**1**)	-	+++	2.37 ± 0.2	1.77 ± 0.1
Quercitrin (**2**)	-	+++	2.12 ± 0.1	1.13 ± 0.1
Myricetrin (**3**)	-	+++	3.85 ± 0.3	1.17 ± 0.1
Trolox[d]	-	-	0.63 ± 0.1	4.72 ± 0.1

[a]Spots intensity; (+) weak, (++) moderate, (+++) strong, and (–) not active.
[b]Spots of the hexane extracts on TLC plate, developed by hexane/ethyl acetate (87:13).
[c]Spots of the hexane and ethanol extracts; ethyl acetate, *n*-butanol, hydromethanol fractions, and flavonols **1**-**3** on TLC plate, developed by ethyl acetate/water/formic acid/acetic acid (100:27:11:11).
[d]Positive control.

the medium-polarity fractions.

The ethyl acetate fractions from stem barks and green fruits exhibited the best activity against $ABTS^{\bullet+}$, with IC_{50} values (µg/ml) of 2.10 ± 0.1 and 4.53 ± 0.7, respectively, and most ethyl acetate fractions from roots and branches displayed a FRSA considered moderate, which IC_{50} value of 10.5 ± 0.7 and 33.0 ± 2.0 µg/ml. On the other hand, *n*-butanol fraction from stem barks showed potent FRSA on $DPPH^{\bullet}$, with IC_{50} value of 4.10 ± 0.3 µg/ml.

Among all the analyzed samples by bleaching experiment on β-carotene, a strong antioxidant effect was found mainly in the ethyl acetate fractions from green fruits and stem barks, and *n*-butanol fraction from stem barks. Altogether, these results indicated a clear positive correlation between $ABTS^{\bullet+}$/$DPPH^{\bullet+}$ scavenging activity and antioxidant properties of these fractions as shown on Table 1.

Higher plants are known to provide a diverse range of secondary metabolites (Fabricant and Farnsworth, 2001). In recent years, flavonoids have been widely recognized as a major class of secondary metabolites with antioxidant properties due to their ability to scavenge free radicals (Havsteen, 2002). Additionally, phytochemical study of stem barks furnished three flavonols responsible for the observed FRSA of ethyl acetate fraction, which were identified as myricetin (**1**), quercitrin (**2**) and myricetrin (**3**), exhibiting IC_{50} values of 2.37 ± 0.2, 2.12 ± 0.1 and 3.85 ± 0.3 µg/ml on $ABTS^{\bullet+}$, respectively. $DPPH^{\bullet}$ scavenging activity of 1-3 has been evaluated as well, showing IC_{50} values of 1.77 ± 0.1, 1.13 ± 0.1 and 1.17 ± 0.1 µg/ml, respectively. The molecular structures of flavonols 1-3 were illustrated in Figure 1.

Compounds **1-3** are flavonols (or 3-hydroxy-flavone derivatives) of widespread occurrence in nature whose medicinal properties have been extensively demonstrated in the literature, especially the FRSA (Lemanska et al., 2001; Cai et al., 2006). Their antioxidant properties have

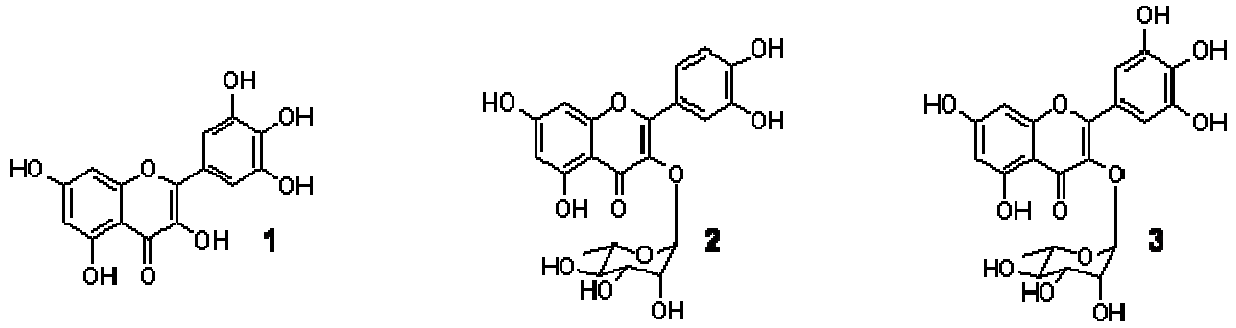

Figure 1. Molecular structures of myricetin (**1**), quercitrin (**2**) and myricetrin (**3**); three flavonols isolated from ethyl acetate fraction of *P. nitens* stem barks.

been attributed to their capacity to scavenge free radicals generated in aqueous phase, increasing the resistance of lipids, proteins, and nucleic acids against oxidation (Pietta, 2000). Moreover, the electroactivity of flavonols 1-3 is due to the presence of structural features such as *ortho*-dihydroxy groups (catechol group on ring B), *α*,*β*-unsaturated carbonyl moiety and *β*-hydroxyketone groups, which are responsible for enhancement of the radicalar stabilization after the initial oxidation steps (Bors et al., 1990).

It may be concluded from the results of this study that *P. nitens* have potential antioxidant activity based on scavenging $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, as well as by bleaching β-carotene assay. Furthermore, ethyl acetate fraction from stem barks could be an important source of potent radical scavengers, useful for developing of novel antioxidant agents. Three flavonoids, myricetin (**1**), quercitrin (**2**), and myricetrin (**3**) have been isolated and identified in the more electroactive fraction, which could be responsible, at least in part for the observed FRSA. In view of these findings, further chemical and pharmacological investigations to identify others secondary metabolites and to evaluate the potential of this species as an antioxidant *in vivo* are recommended.

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