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Free-radical Scavengers and Antioxidants from *Peumus boldus* Mol. ("Boldo")

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The dry leaves of *Peumus boldus* (Monimiaceae) are used in infusion or decoction as a digestive and to improve hepatic complains. Preliminary assays showed free-radical scavenging activity in hot water extracts of boldo leaves, measured by the decoloration of a methanolic solution of the 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH). Assay-guided isolation led to the active compounds. Catechin proved to be the main free-radical scavenger of the extracts. Lipid peroxidation in erythrocytes was inhibited by boldo extracts and fractions at 500 μg/ml with higher effect for the ethyl acetate soluble and alkaloid fractions. The IC_{50} for catechin and boldine in the lipid peroxidation test were 75.6 and 12.5 μg/ml, respectively. On the basis of dry starting material, the catechin content in the crude drug was 2.25% while the total alkaloid calculated as boldine was 0.06%. The activity of boldine was six times higher than catechin in the lipid peroxidation assay. However, the mean catechin:total alkaloid content ratio was 37:1. The relative concentration of alkaloids and phenolics in boldo leaves and their activity suggest that free-radical scavenging effect is mainly due to catechin and flavonoids and that antioxidant effect is mainly related with the catechin content. The high catechin content of boldo leaves and its bioactivity suggest that quality control of *Boldo folium* has to combine the analysis of catechin as well as their characteristic aporphine alkaloids.

Keywords: *Peumus boldus*; Catechin; Boldine; Lipid peroxidation; Free-radical scavengers

INTRODUCTION

The crude drug *Boldo folium* consist of the dry leaves of *Peumus boldus* Molina (Monimiaceae). Boldo is used in infusion or decoction as a digestive and to improve hepatic complains.[1,2] Several studies have been undertaken to relate the activity of the crude drug with the alkaloid boldine, one of its characteristic secondary metabolites. The alkaloid concentration in boldo leaves is not very high, but total phenolics account for much higher figures. Preliminary assays showed free-radical scavenging activity in hot water extracts of boldo leaves, measured by the decoloration of a methanolic solution of the 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) and inhibition of lipid peroxidation in erythrocytes. The aim of the present work was to identify the main free-radical scavenger, xanthine oxidase (XO)- and lipid peroxidation inhibitory compounds from *P. boldus* leaves.

MATERIALS AND METHODS

Boldo leaves were collected from wild growing trees in the western slopes of the Andes in the road

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TABLE I Percentage of activity relative to the corresponding control induced by boldo extracts on free radical and superoxide anion scavenging, xanthine oxidase and lipid peroxidation inhibition. Results are presented as mean ± standard deviation

Sample	Decoloration of DPPH			Scavenging of the superoxide anion	XO inhibition	Inhibition of lipid peroxidation	% Yield*
Sample concentration (μg/ml)	100	50	10	50	50	500	
Extract/compound							
Boldo, crude water extract	77 ± 2	61 ± 3	43 ± 1	73 ± 4	NS	27.7 ± 6.5	17.58
Retained in Amberlite							
EtOAc-soluble	72 ± 3	66 ± 3	54 ± 4	81 ± 3	NS	44.1 ± 12.2	2.45
Water-soluble	73 ± 3	56 ± 3	44 ± 3	77 ± 5	NS	16.8 ± 7.6	75.20
Basic extract (alkaloids)	54 ± 4	39 ± 4	13 ± 3	NS	NS	35.0 ± 2.7	
Alkaloid-free extract	47 ± 2	40 ± 3	34 ± 2	37 ± 4	NS	15.2 ± 6.9	
Not retained in Amberlite	12 ± 3	NS	NS	NS	17 ± 2	3.1 ± 4.2	4.05
		IC_{50} (μg/ml)				IC_{50} (μg/ml)	
Catechin		2.39–3.53		88 ± 3	NS	75.6	2.25
Boldine		4.01–5.35		NS	35 ± 2	12.5	0.06

*Percentage yields from dry starting material. NS: not significant.

to Armerillo, VII Region, Chile, during spring 2000. The air-dried coarsely ground leaves (2 kg) were extracted two times with boiling water (1 × 15 l; 1 × 10 l) for 15 min. After filtration, 20 l of a dark brown solution was obtained. The infusion was passed through Amberlite XAD 7 and XAD 16 columns. The columns were rinsed with water and washed with MeOH:H_2O 1:1 (2 l), MeOH (4 l) and acetone (4 l). The eluates were concentrated under reduced pressure and partitioned with EtOAc to afford an EtOAc-soluble and a water-soluble fraction. Representative samples were lyophilized to determine the w/w yields in terms of dry starting material.

Most of the free-radical scavenging effect was found in the extracts obtained after elution of the Amberlite-retained part of the decoction (Table I). About 21 g of the EtOAc-soluble part of the Amberlite-retained extract was permeated in a Sephadex LH-20 column and eluted with MeOH to obtain 77 fractions of 5 ml each. After comparison of the TLC pattern, fractions were pooled into seven groups. Fractions were assessed for their ability to decolorize a DPPH solution as well as for their capacity to scavenge superoxide anion. In addition, fractions were assessed towards the enzyme XO. A quantity of 250 mg of fraction 5 was further purified in Sephadex LH-20 (37 cm; 2.5 cm i.d.) using methanol as eluent to afford 17 fractions of 5–7 ml each. Pure catechin was obtained from fraction 7 (20.3 mg) of this column and identified by ^{1}H and ^{13}C-NMR spectroscopy, retention time in HPLC with a reference sample, co-chromatography and optical rotation.

A 1-l sample from the water-soluble part of the extract retained in XAD resin was extracted for basic compounds that contained 853 mg of basic and 16.31 g of an alkaloid-free extract.

DPPH Decoloration Assay

The free-radical scavenging effect of the crude extracts and compounds was assessed at 100, 50 and 10 μg/ml by the decoloration of a methanolic solution of DPPH (Aldrich), as previously reported.[3,4] The percentage of decoloration was calculated as follows:

$$\text{Percentage of decoloration} = 1 - \frac{\text{Absorbance of compound/extract}}{\text{Absorbance of blank}} \times 100$$

The degree of decoloration indicates the free-radical scavenging efficiency of the substances. A methanolic solution of DPPH served as a control. Values are presented as mean ± standard deviation of three determinations.

Superoxide Anion

The enzyme XO is able to generate $O_2^{\bullet}$ *in vivo* by oxidation of reduced products from intracellular ATP metabolism. The superoxide generated in this reaction sequence reduces the nitro blue tetrazolium dye (NBT), leading to a chromophore with maxima at 560 nm. Superoxide anion scavengers reduce the generation of the chromophore. The activity was measured spectrophotometrically as reported previously.[5,6] Extracts and products were evaluated at 50 μg/ml. Quercetin was used as a reference compound. Values are presented as mean ± standard deviation of three determinations. The percentage of superoxide anion scavenging effect was calculated as follows:

$$\text{Percentage of scavenging activity} = \frac{E - S}{E} \times 100$$

where $E = A - B$ and $S = C - (B + D)$; A is the optical density of the control; B is the optical density of the control blank; C is the optical density of the sample; D is the optical density of the sample blank.

Xanthine Oxidase Activity

Xanthine oxidase derived from cow's milk, xanthine and the standard inhibitor allopurinol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The XO activities with xanthine as substrate were measured spectrophotometrically as previously reported using a Shimadzu UV-160A equipment.[7] The percentage of inhibition was calculated as follows:

$$\text{Percentage of inhibition} = \frac{(\text{Control} - \text{Control blank}) - (\text{Sample} - \text{Sample blank})}{(\text{Control} - \text{Control blank})} \times 100$$

Extracts and products were evaluated at 50 μg/ml. Results are presented as mean ± standard deviation of three determinations.

Peroxidation in Erythrocytes

Studies on erythrocyte lipid peroxidation were carried out as described by De Azevedo *et al.*[8] with slight modifications. Human red blood cells obtained from healthy donors were washed three times in cold phosphate buffered saline (PBS) by centrifugation at 3.500 rpm. After the last washing, cells were suspended in PBS and their density adjusted to 1 mM hemoglobin in each reaction tube. The final cell suspensions were incubated with different concentrations of the test compounds dissolved in DMSO and PBS during 5 min at 37°C. The final concentration of DMSO in the test medium and controls was 1%. After incubation, cells were exposed to *tert*-butylhydroperoxide (1 mM) during 15 min to 37°C under shaking. After treatment, lipid peroxidation was determined indirectly by the TBARs formation as described previously.[8] Results are expressed as percentage of inhibition compared to controls as described by Mathiesen *et al.*[9] Each determination was repeated four times. To calculate the IC_{50} values (concentration that produces a 50% inhibitory effect on the TBARs formation), the results were transformed to percentage of controls and the IC_{50} values were graphically obtained from the dose-response curves.

The percentage of inhibition of the formation of TBARs (% inhibition of lipid peroxidation) was calculated as follows:

$$\%\ \text{Inhibition of lipid peroxidation} = \frac{(A_1 - A_t)}{(A_1 - A_2)} \times 100$$

where A_1, A_2 and A_t are the absorbance values at 535 nm for the unprotected samples, the blanks and the test samples, respectively.

HPLC Analyses of Phenolics

All analyses were performed using a Merck–Hitachi equipment consisting of a L-6200 pump, a L-4000 UV detector and D-2500 chromato-integrator. Screening of flavonoids and phenolic acids in the sample was carried out by the methodology of Häkkinen *et al.*[10] using a 250 mm × 4 mm Lichrospher RP 18 column. The solvent system used to assess the presence of phenolic compounds was the following. Solvent A: 50 mM ammonium dihydrogen phosphate, pH 2.6; Solvent B: 0.20 mM *ortho*-phosphoric acid, pH 1.5; Solvent C: 20% solvent A in 80% acetonitrile. The gradient elution program was as reported previously.[10]

Under these conditions, the reference compounds (Sigma, USA and Merck, Germany) eluted with the following R_t (min): gallic acid (15.43), catechin (38.38), epicatechin (41.98), chlorogenic acid (44.64), epigalocatechin 3-*O*-gallate (47.62), rutin (54.80), quercetin-3-*O*-β-galactoside (54.97), ellagic acid (55.50), benzoic acid (56.43), quercetin (61.41), apigenin (63.11), kaempferol (63.52). Total phenolic content of the plant and extracts was determined by the Folin–Ciocalteau technique using a calibration curve with tannic acid and gallic acid.[11,12] Total flavonoid content was estimated by the aluminum chloride method.[13] Catechin content was determined by HPLC using a calibration curve with reference compound. Total alkaloid content in the extracts was determined spectrophotometrically according to Franz and Koehler.[14]

TABLE II Total alkaloid, flavonoid, phenolics and catechin content in boldo (*P. boldus*) leaf extracts (g% from dry extract). Results are presented as mean ± standard deviation

Extract	Alkaloids	Total phenolics		Flavonoids	Catechin*
		TA	GA		
Hot water (decoction)	0.34 ± 0.05	14.4 ± 0.3	15.2 ± 0.6	0.12 ± 0.02	12.81 ± 0.32
Retained in Amberlite, EtOAc soluble	0.22 ± 0.01	30.6 ± 0.1	34.6 ± 0.2	0.50 ± 0.03	16.99 ± 0.10
Retained in Amberlite, water-soluble	0.45 ± 0.01	31.6 ± 0.1	36.1 ± 0.1	0.25 ± 0.01	11.53 ± 0.06
Retained in Amberlite, alkaloid-free extract	0.06 ± 0.01	31.6 ± 0.02	36.1 ± 0.1	0.21 ± 0.02	11.10 ± 0.01
Not retained in Amberlite, total	0.04 ± 0.01	13.2 ± 0.1	14.3 ± 0.1	<0.01	<0.01

TA, tannic acid; GA, gallic acid. *By HPLC with a standard curve.

Total alkaloid, flavonoid, phenolics and catechin content in boldo (*P. boldus*) leaf extracts (g% from dry extract) is presented in Table II.

Catechin (3′,4′,5,7-tetrahydroxyflavan-3-ol): MS (m/z): 290.07904 (calc. for $C_{15}H_{14}O_6$: 290.07904); $[\alpha]_D^{20°C}$: −18.58 ($c = 0.99$; MeOH). ^{1}H-NMR (200 MHz, MeOH-d_4, δ in ppm): 4.74 d (H-2); 3.98 m (H-3); 2.81 dd (H-4); 2.66 dd (H-4a); 6.38 s br (2H; H-6 and H-8); 6.82 d (H-2′); 6.78 d (H-5′); 6.68 dd (H-6′); J (Hz): 2,3 = 6.6; 4,4a = 15.6; 4,3 = 4.8; 4a,3 = 8.5; 2′,6′ = 1.7; 5′,6′ = 8.1. ^{13}C-RMN (50 MHz, MeOH-d_4, δ in ppm): 80.89 d (C-2); 66.27 d (C-3); 25.38 t (C-4); 111.85 s (C-4a); 144.69 s (C-5); 114.23 d (C-6); 144.00 s (C-7); 107.93 d (C-8); 144.76 s (C-8a); 130.67 s (C-1′); 117.97 d (C-2′); 142.88 s (C-3′); 132.68 s (C-4′); 115.01 d (C-5′); 118.43 d (C-6′).

Statistical Analysis

Results are expressed as the mean of the percentage of inhibition relative to the corresponding control ± standard deviation. Statistical significance was determined by Student's t-test (unpaired t-test), with the level of significance set at $p < 0.05$. Absorbance values with $p \geq 0.05$ were considered as not significant (NS).

RESULTS

Hot water extracts of boldo leaves showed free-radical scavenging activity in the DPPH decoloration test, superoxide anion and lipid peroxidation in erythrocytes. In the DPPH assay, the most active fractions were the Amberlite-retained EtOAc-soluble and water-soluble fractions. In the superoxide anion test, the same EtOAc and water-soluble Amberlite-retained fractions were the most active fractions with scavenging activities of 81 and 77%, respectively. Under our experimental conditions, the standard compound quercetin showed a scavenging activity of 84–88% at 50 μg/ml. The extracts were inactive towards the enzyme XO at 50 μg/ml while boldine showed a mild inhibition on the enzyme. Results are summarized in Table I.

In the lipid peroxidation assay, highest activity was found in the EtOAc-soluble fraction of the Amberlite-retained extract (44.1%) as well as in the alkaloid extract (35%). The water-soluble Amberlite-retained fraction containing both phenolics and alkaloids as well as the same fraction after alkaloid extraction presented similar activities (16.8 and 15.2%, respectively). The alkaloid content in the water-soluble Amberlite-retained fraction was quite low (0.45%) compared with total phenolics (31.6%), while for the same fraction after alkaloid extraction the figures were 0.06 and 31.6%, respectively (Table II).

Fractionation of the crude "boldo" extract by means of Amberlite resins and further elution allowed the concentration of the phenolic material in the Amberlite-retained fraction. The activity of the water-solubles that were not retained in Amberlite was marginal in the DPPH test at 100 μg/ml, inactive in the superoxide anion and lipid peroxidation test and slightly active towards the XO at 50 μg/ml. After passing through the resin, the solution that was not retained contained very low concentrations of flavonoids and catechin.

Total alkaloid and phenolic content of boldo extracts on a dry extract basis (Table II) indicates that the concentration of alkaloids is low (0.34%), while total phenolics in the hot water extract ranges from 14.4 to 15.2% depending on whether results are shown with reference to tannic or gallic acid. After partition in Amberlite resin, the total phenolic content increases in the Amberlite-retained fractions.

DISCUSSION

The free-radical scavenger and antioxidant properties of boldine have been reported by Jimenez *et al.*[15] using a methodology based on the free radical-induced erythrocyte lysis. The authors report that in addition to its antioxidant effects, boldine displays time-dependent strong cytoprotective properties against chemically induced hemolytic damage. Further experiments on boldine have been performed by Bannach *et al.*[16] using

tert-butylhydroperoxide-induced damage to isolated hepatocytes. At 200 μmol/l, boldine fully cytoprotected and completely prevented the peroxidation induced by *tert*-butylhydroperoxude at concentrations equal to or lower than 0.87 mmol/l. Using rat hepatic microsomes, lipid peroxidation was inhibited by boldine with IC_{50} of 0.015 mM.[17] Under our experimental conditions, using human erythrocytes the IC_{50} of boldine was 0.038 mM.

A careful study on the antioxidant properties of boldine in systems under lipid peroxidation conditions indicates a high activity of the alkaloid towards free radicals.[18] The effect, however, is also related to the radical species as shown with the superoxide anion, where boldine was inactive at 50 μg/ml while the phenolic catechin presented a high scavenging effect (88%) at the same concentration.

In a review on boldo and boldine, Speisky and Cassels[19] focus on the antioxidant properties of the alkaloid in both biological and non-biological systems. Other components like phenolics in the plant are not covered in the review. The hepatoprotective and anti-inflammatory activity of an hydroalcoholic extract of boldo has been published by Lanhers *et al.*[20] These authors report that boldine can be implicated in the hepatoprotective activity, but not in the anti-inflammatory effect.

Other biological activities reported for boldine comprises its effect on rat ileum,[21] intestinal transit and experimental colitis,[22,23] antioxidant activity in fish oil,[24] antipyretic and anti-inflammatory effect.[25]

Little is known on the role of compounds other than alkaloids in the biological activity of boldo leaves. Most bioactivity studies have been carried out with crude extracts and with the alkaloid boldine, which is not the main compound in the leaves.

The boldo alkaloids have been intensively investigated and several reports refer to their composition,[26–29] their biosynthesis in tissue culture[30,31] and quantification on biological fluids.[32] Flavonoids have been also reported from *P. boldus*.[33]

The antioxidant effect of catechin on lipid peroxidation has been reported by Gorelik and Kanner.[34] The authors informed that catechin at 500 μM decreased by 90% membrane lipid peroxidation and by 50% oxymyoglobin oxidation. Using erythrocyte membrane, Liao and Yin[35] compared the antioxidant effect of several flavonoids, including catechin. The antioxidant activity followed the order catechin > epicatechin > rutin > quercetin > myricetin. Catechins and quercetin are peroxyl and hydroxyl free-radical scavengers and show protective effects against lipid peroxidation.[36]

It has been reported that catechin is an inhibitor of COX-1 and COX-2. These enzymes are involved in the prostaglandin biosynthesis.[37] Prostanglandins are important mediators in the inflammatory process, and the inhibition of COX is involved in the way of action of many anti-inflammatory drugs, although the *in vitro* effects may not have a correlation *in vivo*. In this assay, the IC_{50} of catechin was 80 μM.

The low alkaloid content in boldo leaves and their activity pointed out that the free-radical scavenging effect of the plant is mainly due to their phenolic compounds. The main phenolic was identified as catechin and its bioactivity relates well with the observed for boldo extracts. The high catechin content of boldo leaves and its bioactivity suggest that quality control of *Boldo folium* has to combine the analysis of catechin as well as their characteristic aporphine alkaloids.

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