Antioxidant Metabolites from *Limonium brasiliense* (Boiss.) Kuntze

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A free radical scavenging activity guided fractionation of the polar extract from roots of *Limonium brasiliense* (Plumbaginaceae) led to the isolation of five active compounds including: myricetin 3-*O*-α-rhamnopyranoside (**1**), (–)-epigallocatechin 3-*O*-gallate (**2**), (–)-epigallocatechin (**3**), (+)-gallocatechin (**4**) and gallic acid (**5**). These and other chemical constituents are reported for the first time for this species. The characterization of these compounds was achieved by spectroscopic methods (^{1}H NMR, ^{13}C NMR and UV).

Key words: Limonium brasiliense, Flavonoids, Antioxidants

Introduction

Limonium brasiliense (Boiss.) Kuntze (Plumbaginaceae) is a perennial herb, known by its common name "guaycurú", that grows mostly in saline soils, distributed in Argentina, Uruguay and in the South of Brasil. Infusion from the roots is popularly used in the treatment of hemorrhage, menstrual disorders, rheumatism and it is believed to have cardioprotective properties (Gupta, 1995). The alcoholic extracts from the roots have showed bacteriostatic activity, anti-inflammatory activity and ocytocin and bradykinin antagonism which could explain its action in patients with dysmenorrhea, amenorrhea and metrorrhagia (Jahns and Crescente, 1976). Previous chemical studies reported the presence of tannins, leukoanthocyanins, hydrocyanic acid and ellagic acid in the roots (Medina *et al.*, 1977; Ragonese and Milano, 1984).

In the course of our studies of medicinal plants from the southern region of Argentina we found that the methanolic extract from the roots of *Limonium brasiliense* showed antioxidant activity as it was observed by the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH).

Oxidation is well known to be the major cause of material degradation. In food, the process of lipid autoxidation and development of rancidity involves a free radical chain mechanism (Shahidi, 1997). *In vivo*, free radical initiated autoxidation of cellular membrane lipids can lead to cellular necrosis. It is well recognized that oxygen reactive species, in particular free radicals, are involved in a variety of pathological conditions such as cancer, cardiovascular disease, arteriosclerosis and neurodegenerative diseases (Haliwell, 1992; Markesbery and Carney, 1999; Schroeter *et al.*, 2000). Even aging may be considered as the result of deleterious free radical reactions which occur throughout cells and tissues (Maxwell, 1995). For all these reasons the study of new sources of natural antioxidants is receiving increasing attention

In the present work we are reporting the antioxidant metabolites isolated from the polar extract of the roots of *L. brasiliense*, which have not been previously reported from this plant. Besides, other chemical constituents of the roots like anthocyanins isolated from the MeOH/HCl (1%) extract are reported.

Material and Methods

General

^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 or Acetone-d_6 using TMS as internal standard on a Bruker ARX 300 multinuclear spectrometer at 300 MHz and 75 MHz, respectively. UV spectra were recorded on a GBC Spectral UV-VIS spectrophotometer.

Silica gel 60 (70–230 mesh, Fluka) was used for column chromatography. Thin layer chromatography was performed using polyamide plates (MN-Polyamid-DC6, Macherey, Nagel & Co) with solvent system A (nitromethane/acetic acid/2-butanone 5:1:10 v/v/v) or B (benzene/2-butanone/

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methanol 5:2:3 v/v/v). The spots were visualized using UV light (254 and 366 nm) and spraying with ethanolic $AlCl_3$ solution (5%). For the free radical scavenging activity assay 2,2-diphenyl-1-picrylhydrazyl was purchased from Sigma.

Plant material

Roots of *Limonium brasiliense* were collected at San Martín, La Pampa, Argentina, in October 2002. A voucher specimen was identified by Ing Sergio Lamberto and was deposited in the Herbario Regional, Departamento de Agronomía, Universidad Nacional del Sur (BB3776–3993).

Extraction and isolation

Dried roots (247 g) were milled and extracted with refluxing methanol overnight (3 × 1 l). The combined methanolic extracts were evaporated *in vacuo* to yield 22.9 g of a brown residue that was dissolved in water and partitioned with chloroform and ethyl acetate. After solvent evaporation under reduced pressure the ethyl acetate extract (1.3 g) was chromatographed on a silica gel column using hexane, ethyl acetate and methanol mixtures of increasing polarity. All collected fractions **1**–**15** (100 ml each) were evaluated with DPPH assay on TLC. Fractions **7**, **8**, **9**, **10**, and **11**, which elicited radical scavenging activity, were separately submitted to preparative TLC on polyamide plates using solvent system A and/or B. Active compounds were identified as myricetin 3-*O*-α-rhamnopyranoside (**1**) (48.8 mg), (−)-epigallocatechin 3-*O*-gallate (**2**) (13.0 mg), (−)-epigallocatechin (**3**) (21.1 mg), (+)-gallocatechin (**4**) (22.0 mg) and gallic acid (**5**) (8.1 mg). The structures of the isolated compounds were determined on the basis of their ^1H and ^{13}C NMR spectra and confirmed by comparison with literature data (Agrawal, 1989; Davis *et al.*, 1996; Markham *et al.*, 1978).

DPPH assay

The antioxidant activity was evaluated by the ability as free radical scavenger of extracts, fractions and/or pure compounds. The preliminary test was performed with a rapid TLC screening method using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). Analytical TLC on polyamide plates was developed under appropriate conditions after application of 5 μl of each test compound solution (1 mg/ml), dried and sprayed with DPPH solution (0.2%, MeOH). 5 min later active compounds appeared as yellow spots against a purple background. The purple stable free radical 2,2-diphenyl-1-picrylhydrazyl was reduced to the yellow colored diphenylpicryl hydrazine. Quercetin was used as positive control.

The spectrophotometric assay was carried out as follows: 200 μl of a methanolic solution of the test compounds or extract were added to 3.2 ml of a 0.004% DPPH solution in MeOH. Seven concentrations, ranging from 1 to 100 μM, were prepared for each sample and analyzed in triplicate. 3.2 ml of MeOH plus 200 μl of each compound solution were used for blank solutions. 3.2 ml of 0.004% DPPH solution plus 200 μl of MeOH were used for negative control. The absorbance at 517 nm was determined after 30 min of incubation and the percentage of DPPH reduction was calculated taking into account the absorbance of the blank solutions and the negative control. Quercetin was used as reference compound under the same experimental conditions. Results are summarized in Table I.

Anthocyanins

Roots of *L. brasiliense* (300 g) were extracted 24 h with MeOH/HCl (1%) at room temperature in the dark. This extract was evaporated to dryness under inert atmosphere and chromatographed on Whatman 3MM paper with *n*-BuOH/AcOH/H_2O (4:1:5 v/v/v) and 15% AcOH. Acid hydrolysis and oxidative degradation of the isolated pigments revealed a common aglycone, delphinidin, and glycosidation with rhamnose and glucose. With the aid of UV spectra, chromatographic methods and comparison with authentic samples, these anthocyanins were identified as delphinidin 3-rhamnoside (**6**), delphinidin 3-glucoside (**7**) and delphinidin 3,5-diglucoside (**8**).

Results and Discussion

The presence of antioxidant metabolites in the methanolic extract from the roots of *L. brasiliense* was detected in a preliminary screening of radical scavenging activity against DPPH in an autographic assay (Bors *et al.*, 1992). An activity guided fractionation of this extract led us to the isolation of the active compounds, one flavonoid identified as myricetin 3-*O*-α-rhamnopyranoside (**1**), three flavan-3-ols, (−)-epigallocatechin 3-*O*-gallate (**2**), (−)-epigallocatechin (**3**) and (+)-gallo-

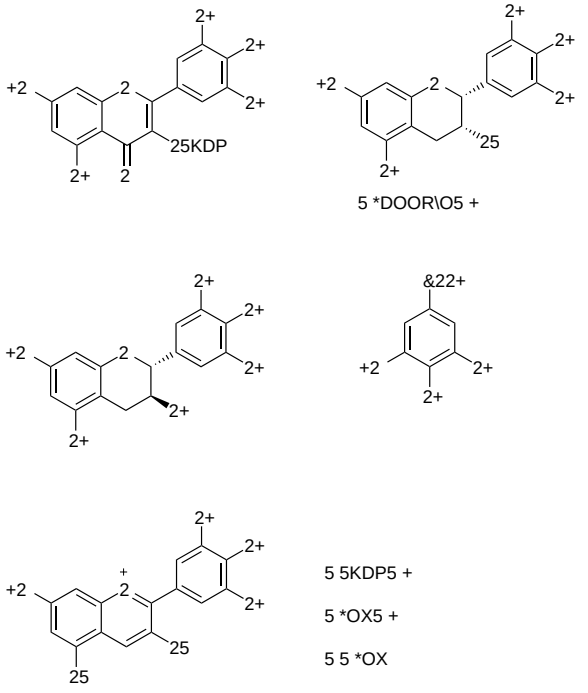

Fig. 1. Compounds isolated from the roots of *L. brasiliense*: myricetin 3-*O*-α-rhamnopyranoside (**1**), (−)-epigallocatechin 3-*O*-gallate (**2**), (−)-epigallocatechin (**3**), (+)-gallocatechin (**4**), gallic acid (**5**), delphinidin 3-rhamnoside (**6**), delphinidin 3-glucoside (**7**) and delphinidin 3,5-diglucoside (**8**).

catechin (**4**), and gallic acid (**5**) (Fig. 1). These compounds were purified by chromatographic methods and identified by comparison of their ^{1}H and ^{13}C NMR data with those reported in the literature (Markham *et al.*, 1978; Agrawal, 1989; Davis *et al.*, 1996). The major constituent of the active fractions, myricetin 3-*O*-α-rhamnopyranoside (**1**) have been, as previously reported, also isolated from other species of the genus *Limonium*, *Limonium sinense* (Lin and Chou, 2000) and *Limonium* cv. 'Gold Coast' (Asen and Plimmer, 1972). (−)-Epigallocatechin 3-*O*-gallate (**2**), (−)-epigallocatechin (**3**) and gallic acid (**5**) are also present in *Limonium sinense* (Lin and Chou, 2000). These polyphenolic compounds are ones of the major constituents of the green tea leaves extract and are well recognized to be responsible for the antioxidant properties of tea. They are effective scavengers for radicals generated in aqueous phases and also against the propagation lipid peroxyl radicals (Salah *et al.*, 1995; Hirano *et al.*, 2001). (−)-Epigallocatechin (**3**) and its gallate ester **2** have also displayed antitumour activity against human cancer cell lines (Yang *et al.*, 1998). (−)-Epigallocatechin 3-*O*-gallate (**2**) have exhibited potent inhibitory activities in the replication of Herpes Simplex Virus Type-1 (Lin *et al.*, 2000). Finally, gallic acid (**5**) has been identified as the active component of the water extract of *Limonium wrightii* with a strong free radical scavenging action (Aniya *et al.*, 2002).

In the DPPH assay on TLC active compounds appear as yellow spots against a purple background. In this case, the methanolic extract and its major constituent, myricetin 3-*O*-α-rhamnopyranoside (**1**), seemed to be more active than quercetin, used as a positive control under the same experimental conditions. Then, a spectrophotometric assay was carried out in order to evaluate the radical scavenging ability by measuring the percentage of reduction of a 0.004% DPPH solution. Thus, quercetin resulted to be more active (IC_{50} = 20.7 μM) than myricetin 3-*O*-α-rhamnopyranoside (IC_{50} = 40.0 μM) under the chosen experimental conditions. The methanolic extract, rich in myricetin 3-*O*-α-rhamnopyranoside (**1**) and polyphenolic compounds (**2**–**5**) elicited a high free radical scavenging activity (IC_{50} = 20 μg/ml) (Table I).

In the MeOH/HCl (1%) extract from the roots of *L. brasiliense* the presence of anthocyanins was detected. Even if anthocyanins are usually found in flowers, fruits and aerial parts of a plant they are seldom detected in roots. These pigments were isolated and purified from the MeOH/HCl extract by paper chromatography rendering three predominant compounds that were identified by chemical methods (acid hydrolysis, oxidative degradation), UV spectroscopy and chromatography (Maza and Minatti, 1993), as well as by comparison with authentic samples, as delphinidin 3-rhamnoside (**6**), delphinidin 3-glucoside (**7**) and delphinidin 3,5-diglucoside (**8**) (Fig. 1). These pigments

Table I. DPPH scavenging activity of compound **1**, methanolic extract and quercetin.

	DPPH activity IC_{50} [μM]
Myricetin 3-*O*-α-rhamnopyranoside (**1**)	40.0
Quercetin	20.7
Methanolic extract[a]	20.0[b]

[a] Mixture of polyphenolic compounds.
[b] In μg/ml.

have also been identified in other species of the Plumbaginaceae family (Harborne, 1967).

This study shows that roots of *L. brasiliense* are a rich source of compounds with antioxidant activity like **1**–**5**. This may support the popular use of the infusion from these roots as a cardioprotective natural dietary supplement. It is well recognized that polyphenolic compounds of the higher plants, because of their antioxidant ability, contribute to the prevention of cardiovascular disease (Salah *et al.*, 1995).

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