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Short Report

Flavonol Robinobiosides and Rutinosides from *Alternanthera brasiliana* (Amaranthaceae) and their Effects on Lymphocyte Proliferation *In Vitro*

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O extrato da espécie medicinal *Alternanthera brasiliana* Kuntze forneceu seis derivados di- e triglicosilados de kaempferol e quercetina. As estruturas foram elucidadas com base em RMN de ^{1}H e ^{13}C. Os seis flavonóides são inéditos no gênero. Kaempferol 3-*O*-robinobiosídeo e kaempferol 3-*O*-rutinosídeo inibiram mais eficientemente a proliferação de linfócitos humanos *in vitro*.

The extract of the medicinal species *Alternanthera brasiliana* Kuntze afforded six di- and triglycosyl kaempferol and quercetin derivatives. Their structures were elucidated based on the ^{1}H- and ^{13}C-NMR data and are reported here for the first time in this genus. Kaempferol 3-*O*-robinobioside and kaempferol 3-*O*-rutinoside significantly inhibited the human lymphocyte proliferation *in vitro*.

Keywords: *Alternanthera brasiliana*, Amaranthaceae, flavonol robinobiosides, flavonol rutinosides, lymphocyte proliferation

Introduction

Alternanthera brasiliana Kuntze (Amaranthaceae) is a herbaceous plant used against inflammation, cough and diarrhoea in Brazilian popular medicine.[1] In a search for some action of this plant against inflammatory cells of the immune system, it was shown that aqueous or ethanolic *A. brasiliana* leaf extracts are able to block human mitogen-induced lymphocyte proliferation, without any toxic effect.[2] Different biological activities have been established for *A. brasiliana* species; [2-4] however, this is the first chemical study of its secondary metabolites.

Results and Discussion

Bioassay-guided fractionation of a water-soluble phase of an ethanolic leaf extract (EE) was carried out with the aim of identifying compounds responsible for the antiproliferative effect of the crude extract on lymphocytes.

Six flavonoids were identified: kaempferol 3-*O*-robinobioside-7-*O*-α-L-rhamnopyranoside or robinin (**1**), quercetin 3-*O*-robinobioside-7-*O*-α-L-rhamnopyranoside or clovin (**2**), quercetin 3-*O*-robinobioside (**3**), kaempferol 3-*O*-robinobioside (**4**), kaempferol 3-*O*-rutinoside-7-*O*-α-L-rhamnopyranoside (**5**) and kaempferol 3-*O*-rutinoside (**6**). Their structures were elucidated by ^{1}H- and ^{13}C-NMR data (^{1}H-^{1}H COSY, APT, HETCOR) and agree with literature data.[5-9]

A. brasiliana contains mainly 3-*O*-robinobioside derivatives of kaempferol and quercetin. The kaempferol skeleton was present in four of the six flavonoids isolated. Additional glycosylation was observed at C-7 for **1**, **2** and **5**. Only two flavonoids (**5**, **6**) showed a rutinosyl instead of a robinobiosyl moiety at C-3. These results demonstrate that the 1→6 galactopyranosyl-rhamnopyranosyl linkage is prevalent among the *A. brasiliana* flavonol glycosides. To the best of our knowledge, the two glycosyl flavones isolated from *A. phyloxeroides* are the only flavonoids previously reported for this genus.[10,11]

Although not widespread in nature, flavonol robinobiosides have been isolated from different plant species, particularly *Strychnos variabilis*.[9,12-13] Recently two spinacetin robinobioside derivatives, one of them belonging to the unusual class of methylene bisflavonoids,

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were described from *Blutaparon portulacoides*, another medicinal species of Amaranthaceae.[14]

Flavonoids **1**-**6** are reported here for the first time for *Alternanthera*. Kaempferol 3-*O*-robinobioside (**4**) and 3-*O*-rutinoside (**6**) inhibited lymphocyte proliferation to a greater extent ($IC_{50} \cong 25\ \mu g\ mL^{-1}$) and were twice as active as the crude extract (Table 1). The anti-inflammatory effects[15] observed *in vivo* can be attributed to the effects of these flavonoids on T-cell function, thereby accounting for the medicinal properties of *A. brasiliana*.

Table 1. Inhibition of the *in vitro* proliferative response of human T-cells by *A. brasiliana* extract (WE) and its flavonoids **1**-**6**

Sample	IC_{50} ($\mu g\ mL^{-1}$)
WE	50
1	50
2	50
3	50
4	**25**
5	100
6	**25**
Azathioprine[a]	0.2

WE= water-soluble extract; [a] positive control.

Experimental

General

^{1}H- and ^{13}C-NMR spectra (DMSO-d_6 signals as internal reference: δ 2.49) were recorded on a Varian Gemini 200 (^{1}H: 200 MHz; ^{13}C: 50 MHz) or in a Bruker DRX-400 (^{1}H-, 400 MHz; ^{13}C-, 100 MHz). $[\alpha]_D$ was measured in a Perkin-Elmer 243B polarimeter; and melting points were obtained using a Kofler apparatus. Reversed-phase chromatography was performed on RP-2 or RP-8 silanized silica (Merck) and size exclusion chromatography on Sephadex LH-20 (Sigma). Eluates were monitored by thin-layer chromatography on Silica 60 F_{254} (Merck) using butanol-acetic acid-water (8:1:1) for development and ceric sulfate solution as detection reagent.

Plant material

A. brasiliana was collected out of flowering season on the University (UFRJ) *campus* (Ilha do Fundão). A voucher specimen (RFA-25 052) was deposited in the herbarium of the Department of Botany (Instituto de Biologia, UFRJ, Brazil).

Extraction and isolation

Fresh purple leaves (2.06 Kg) were macerated with ethanol for a week in the dark. The resulting ethanolic extract afforded a syrupy dark material (EE, 66.1 g) which was washed with distilled water. The water-soluble extract (WE) was lyophilised (28.3 g), re-suspended in water and chromatographed on an RP-2 column with a water/methanol gradient (19 fractions). Four major flavonoid fractions were obtained (H_2O:MeOH ration in parenthesis): F and G (7:3); H (7:3 to 1:1) and J (1:1). Flavonoids **1** and **2** were obtained from fraction F (453 mg) by successive Sephadex LH-20 chromatography (EtOH). Crude **1** (36 mg) was crystallised from ethanol to give pure **1** as a light-yellow solid (27.4 mg), R*f* 0.22: mp 185-187 °C; ^{1}H- and ^{13}C-NMR as described before.[5] After elution of **1**, flavonoid **2** was obtained from the same column as a light-yellow powder (25.5 mg), R*f* 0.18: mp 195-200 °C; $[\alpha]_D$ -66° (c 0.5, EtOH); ^{1}H and ^{13}C-NMR as described before.[6] Concentration of fraction G yielded a precipitate (153 mg) which was separated by centrifugation. A sample (72 mg) was chromatographed on an RP-2 column (H_2O:MeOH 8:2 to MeOH 100%) to obtain flavonoid **5** (yellow powder, 35 mg, R*f* 0.28): mp 195-200 °C, ^{1}H- and ^{13}C-NMR as described before.[7,8] The supernatant (826 mg) of fraction G yielded a crude precipitate (219 mg) which was dissolved in distilled water, and **3** was obtained by freezing and thawing the resulting aqueous solution (yellow powder, 55 mg, R*f* 0.31): mp 193-200 °C; ^{1}H- and^{13}C-NMR as described before.[9] Fraction H (551 mg) was washed with acetone. Concentration of the acetone-soluble phase gave a precipitate (242 mg) that was purified as described for **3** to obtain pure **4** (yellow powder, 57 mg, R*f* 0.35): mp 175-180 °C; ^{1}H and ^{13}C-NMR as reported before.[9] The ethanol-soluble phase of fraction J (484 mg) gave a yellow material (364 mg) which was chromatographed on Sephadex LH-20 (EtOH, 7 fractions). Fraction 2 (33 mg) was dissolved in MeOH:H_2O 3:7 and purified on an RP-8 column. The fraction eluted with 50% MeOH gave flavonoid **6** (yellow crystals, 2.8 mg, R*f* 0.38): mp 178 °C; $[\alpha]_D$ +10.0 (c 0.2, H_2O); ^{1}H-and ^{13}C-NMR as already reported.[7,8]

Lymphocyte proliferation assay

Human peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers by Ficoll-Hypaque density gradient centrifugation. Cells (10^6 cells mL^{-1}) were cultivated in the presence of 5 $\mu g\ mL^{-1}$ phytohemagglutinin (PHA) at 37 °C and 5% CO_2. Dose-response curves were obtained by adding different concentrations of extract, flavonoids and azathioprine to triplicate samples, and inhibition was expressed as IC_{50}. Proliferation was measured by [^{3}H] thymidine incorporation into cellular DNA: 0.5 μCi/well was added 6 h before the end of the culture period

(96 h). Cells were harvested and the radioactivity assessed using a liquid scintillation counter. In controls with PHA alone, proliferation of 42550 ± 1300 cpm.

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