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Flowers from *Kalanchoe pinnata* are a Rich Source of T Cell-Suppressive Flavonoids

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The chemical composition and immunosuppressive potential of the flowers from *Kalanchoe pinnata* (Crassulaceae) were investigated. We found that the aqueous flower extract was more active than the leaf extract in inhibiting murine T cell mitogenesis *in vitro*. Flavonoids isolated from the flower extract were identified and quantitated based on NMR and HPLC-DAD-MS analysis, respectively. Along with quercetin, four quercetin glycosyl conjugates were obtained, including quercetin 3-*O*-β-D-glucuronopyranoside and quercetin 3-*O*-β-D-glucopyranoside, which are described for the first time in *K. pinnata.* All flavonoids inhibited murine T cell mitogenesis and IL-2 and IL-4 production without cell toxicity. This is the first report on the pharmacological activity of flowers of a *Kalanchoe* species, which are not used for curative purposes. Our findings show that *K. pinnata* flowers are a rich source of T-suppressive flavonoids that may be therapeutically useful against inflammatory diseases.

Keywords: *Kalanchoe pinnata*, Crassulaceae, Flowers, Immunosuppressive effect, Lymphocyte proliferation, Quercetin and its glycosides.

Kalanchoe pinnata (Lamarck) Persoon (syn.: *Bryophyllum pinnatum*), belonging to the Crassulaceae family, is a medicinal species from a botanical genus comprising more than a hundred species [1,2]. Leaves from *K. pinnata* (here denominated KP) have long been used for healing wounds and soothing inflammation [3]. Flavonoids are the main phenolic compounds in *Kalanchoe* species. The most comprehensive studies on their chemical composition have focused on the leaves [1,3], whereas very few studies have been devoted to the flowers. As far we know, only flowers of *K. blossfeldiana* and *K. spathulata* have deserved attention for their chemical composition [4,5].

Previously, we demonstrated the potent immunosuppressive effect in mice [6-8] and the presence of bioactive flavonoids such as quercetin 3-*O*-α-L-rhamnopyranoside (quercitrin), kaempferol 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (kapinnatoside), quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside and 4',5-dihydroxy-3',8-dimethoxyflavone 7-*O*-β-D-glucopyranoside [9-11] in KP aqueous leaf extract. Considering the lack of chemical and biological studies on *K. pinnata* flowers, the immuno-modulatory potential and flavonoid content were studied.

The immunomodulatory profile of KP leaves [6-8] led us to use a similar approach on KP flowers for comparison purposes. Thus, the aqueous extracts of leaves and flowers were tested *in vitro* for inhibition of T cell mitogenesis. Both extracts were able to inhibit lymphocyte proliferation in a concentration-dependent manner. However, as shown in Figure 1, the flower extract exhibited a stronger inhibitory effect (IC_{50} = 37.5 μg/mL) than the leaf extract (IC_{50} = 84.9 μg/mL). Interestingly, even though the flower extract was a complex mixture, it was more active than cyclosporin A (IC_{50} = 43.8 μg/mL), one of the most effective immunosuppressive drugs in clinical use. Purification of the flower extract by a combination of precipitation, partition and chromatographic techniques afforded five flavonoids. The flavonol glycosides **2**, **3**, **4** and **5** were obtained as the major components, while flavonol **1** (quercetin) was isolated in a small amount and identified as previously reported [12]. Compounds **3** and **4**, here reported for the first time for KP, were identified as quercetin 3-*O*-β-D-glucuronopyranoside (miquelianin) and quercetin 3-*O*-β-D-glucopyranoside (isoquercitrin), respectively, based on ^{1}H, ^{13}C, and 2-D NMR experiments and by comparison with literature data [12]. Compounds **2** and **5** were identified as quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside and quercitrin, respectively, based on ^{1}H, ^{13}C and 2-D NMR, and comparison with our previous NMR data [10].

The effect of flavonoids **1-5** on T cell mitogenesisis is shown in Figure 2. All of them inhibited lymphocyte proliferation in a concentration-dependent manner. Quercetin **1** exhibited the highest activity (IC_{50} = 2.5 μg/mL), whereas compound **2**, the only diglycosyl flavonoid, was the least active (IC_{50} = 38.8 μg/mL). It is possible that the presence of a disaccharide unit hampered the interaction of compound **2** with the lymphocyte membrane. In addition, flavonoids conjugated with only one carbohydrate unit (compounds **3-5**) exhibited an intermediate activity (Figure 2). These results suggested that increased glycosylation reduced the inhibitory activity of this series of flavonoids, which would agree with a previous study showing reduced binding of glycosylflavonoids to proteins [13].

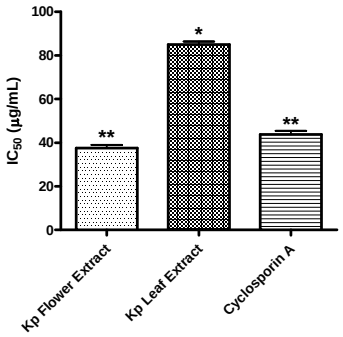

Figure 1: Mitogenic inhibitory activity of extracts from *Kalanchoe pinnata* (KP) flowers and leaves. Murine lymph node cells were stimulated with 5 µg/mL of concanavalin A in the presence of 25, 50 and 100 µg/mL of either the plant extracts or cyclosporin A (positive control). The maximum proliferative response of concanavalin A alone was 10139 cpm. The results were expressed as the drug concentration necessary to produce 50% of the maximum response (IC_{50}). Arithmetic means ± SD (n=3). * p<0.01 in relation to flower extract and cyclosporin A, ** p>0.05 in relation to themselves.

The cytotoxicity of KP extracts and flavonoids from flowers was evaluated through an intracellular lactate dehydrogenase (LDH) release assay. We observed that all samples displayed low cytotoxicity at concentrations much higher than the T cell effective concentration (Table 1). This was particularly true for **1**, **3**, **4** and **5**, which were effective in the range of 2-9 µg/mL. Therefore, the antimitogenic activity observed was likely not due to cytotoxicity. The combined suppressive effect of flavonoids **1-5** could account for the activity of the flower extract on lymphocyte proliferation.

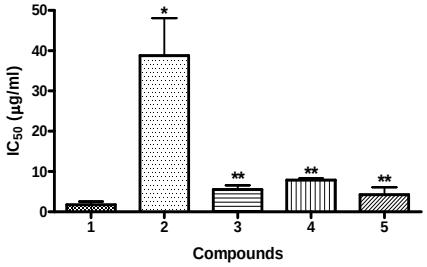

Figure 2: Mitogenic inhibitory activity of flavonoids isolated from *Kalanchoe pinnata* (KP) flower extract. Lymph node cells were stimulated with 5 µg/mL of concanavalin A in the presence of 1, 10 and 100 µg/mL of quercetin (**1**), quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (**2**), quercetin 3-*O*-β-D-glucuronopyranoside (**3**), quercetin 3-*O*-β-D-glucopyranoside (**4**) and quercetin 3-*O*-α-L-rhamnopyranoside (**5**). The maximum proliferative response of concanavalin A alone was 6145 cpm. The inhibitory activity was calculated as in Figure 1. Arithmetic means ± SD (n=3).* p<0.01 in relation to all the other bars, ** p<0.05 in relation to **1.**

Table 1: Cytotoxicity of the crude extracts and flavonoids from the flower extract. Resident macrophages were cultured for 48 h in the presence of leaf extract, flower extract and flavonoids **1-5** at 100 µg/mL. Cytotoxicity was measured by the percentage of specific LDH release in the culture supernatants. Macrophages were also cultured in either the absence of additives (spontaneous release) or with 1% Triton X-100 for maximum release (100%).

Samples	Cytotoxicity (% specific LDH release)
KP leaf extract	6.4 ± 1.2
KP flower extract	0.3 ± 0.4
Quercetin (**1**)	20.3 ± 0
Quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (**2**)	n.d.
Quercetin 3-*O*-β-glucuronopyranoside (miquelianin) (**3**)	1.9 ± 2.7
Quercetin 3-*O*-β-D-glucopyranoside (isoquercitrin) (**4**)	3.0 ± 1.3
Quercetin 3-*O*-α-L-rhamnopyranoside (quercitrin) (**5**)	7.1 ± 0.9

n.d. = not detected

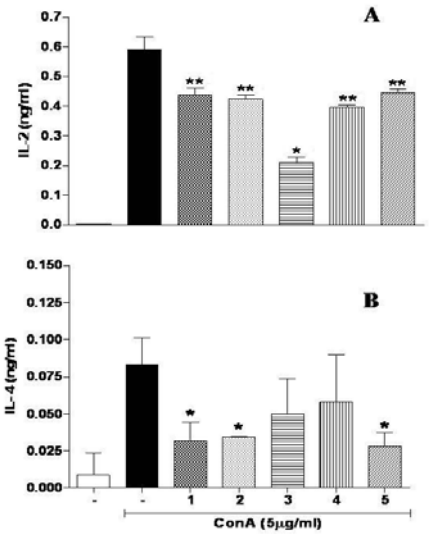

Figure 3: Effect of KP flower flavonoids on cytokine production by lymph node cells. Lymph node cells were stimulated with 5 µg/mL of concanavalin A in the presence of 25 µg/mL of quercetin (**1**), quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (**2**), quercetin 3-*O*-β-D-glucuronopyranoside (**3**), quercetin 3-*O*-β-D-glucopyranoside (**4**) or quercetin 3-*O*-α-L-rhamnopyranoside (**5**). Negative controls were non-treated cells (black column), and positive controls were concanavalin A - stimulated cells (white column). **A)** Production of IL-2 in 48 h cell culture. **B)** Production of IL-4 in 48 h cell culture. Arithmetic means ± SD (n=3). *p<0.01 and **p< 0.05 in relation to positive controls.

The anti-inflammatory and immunosuppressor effects of quercetin have already been proven [14a-14c]. According to Mookerjee *et al*., the reversible lymphoproliferative inhibition effect of flavonoids in response to phytomitogensis is due to their ability to block events triggered by exposure to external stimuli [14d]. T-suppressive activity of flavonoids having a C-2,3-double bond, such as flavonols and flavones, has also been described [14a,14e]. Furthermore, glycosidic substitution(s) abolished or reduced the suppressive effects of their aglycones [14a]. These observations were in agreement with our findings for KP flower flavonoids. Additionally, Cherng *et al*. reported that the glycoside flavonoid rutin (quercetin-3-rutinoside) significantly stimulated the secretion of IFN-γ, but did not enhance the proliferation of human peripheral blood mononuclear cells, indicating that the sugar moiety was critical for the differential immunomodulatory responses between quercetin and its glycoside [14c].

Further experiments were carried out to determine the effect of flavonoids **1-5** on cytokine production by lymph node cells, to explore the mechanisms involved in the T-suppressive activity exerted by KP flower flavonoids. All flavonoids inhibited the production of IL-2, and all except **3** and **4** inhibited the production of IL-4 (Figure 3).

Since IL-2 and IL-4 are classical Th1 and Th2 cytokines, respectively, it can be inferred that both Th1 and particularly Th2-type T cells were suppressed. This was in agreement with our findings showing that orally administered quercitrin (**5**) protected mice against both cutaneous leishmaniasis [9,10] and acute anaphylaxis [6a], two pathologies knowingly driven by Th2 responses. The present finding that not only quercitrin but also compounds **1** and **2** inhibited the production of IL-4 suggested that these flavonoids might have protective effects against those diseases.

Both KP flower and leaf extracts were analyzed by HPLC-DAD-MS to quantify their flavonoid content. As shown in Table 2,

Table 2: Flavonoid content in aqueous *Kalanchoe pinnata* (KP) flower and leaf extracts obtained by HPLC-DAD-MS analysis (254 nm) and expressed as percentage weight/weight (%, w/w) of each flavonoid in the lyophilized extract. Quantification was performed using a calibration curve as explained in the Experimental.

Flavonoids	Retention time (min)	KP flower extract (% w/w)	KP leaf extract (% w/w)
Quercetin (**1**)	44.2	0.12	n.d.
Quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (**2**)	23.1	1.87	2.26
Quercetin 3-*O*-β-D-glucuronopyranoside (miquelianin) (**3**)	20.4	0.79	n.d.
Quercetin 3-*O*-β-D-glucopyranoside (isoquercitrin) (**4**)	19.5	0.21	0.03
Quercetin 3-*O*-α-L-rhamnopyranoside (quercitrin) (**5**)	24.7	0.25	0.32
Overall percentage of flavonoid constituents	---	3.24	2.61

n.d. = not detected.

quercetin arabinosyl-rhamnoside (**2**) was confirmed to be the most abundant flavonoid in both KP flower and leaf extracts [10,15a]. Flavonoids **1** and **3** were present in an amount that could not be quantified by HPLC under the conditions employed.

KP flower extract exhibited a richer flavonoid profile than the leaf extract. Their different flavonoid compositions could explain, at least partially, the different IC_{50} values observed for the antimitogenic activity. The flower extract showed a high flavonoid content of four quercetin bioactive glycosides, besides the minor quercetin **1**, whose immunomodulatory behavior is already well described. This flavonoid mixture could explain the higher activity of KP flower extract in comparison with KP leaf extract.

As mentioned above, only two studies focused on the chemical composition of flowers from *Kalanchoe* species. In a pioneering study, Gaind *et al*. [4] described similar flavonoid contents for leaves and flowers of *K. spathulata*. More than two decades later, Nielsen *et al*. [5] reported the isolation of several anthocyanidins from *K. blossfeldiana* flowers. Interestingly, pink, red and magenta varieties contained relatively high amounts of quercetin derivatives [5].

A novelty of the present study is the T-suppressive activity of flavonoids **2** {quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside}, the most abundant flavonoid in *K. pinnata* flowers, and **3** (quercetin 3-*O*-β-D-glucuronopyranoside). Additionally, flavonoid **2** was the object of a patent on its anti-allergic activity [15b]. IL-4 and IL-13 produced by Th2-type T cells induce the differentiation, activation and *in situ* survival of eosinophils (through IL-5). They also promote the production of high amounts of IgE by B lymphocytes and the growth of mast cells and basophils [16]. Therefore, inhibition of IL-4 by flavonoid **2** may be involved in its reported anti-allergic activity. Whether the flavonoids act directly on mature T cells is not known, but the possibility that they indirectly activate T regulatory cells should not be discarded.

There are few reports in the literature concerning the biological activity of flower-derived compounds [17a,17b,18a]. The present study showed for the first time that the flowers of *K. pinnata* are a rich source of non-cytotoxic immunomodulatory substances. Due to the preferential Th2-suppressive action of flavonoids **1**, **2** and **5**, they may have potential therapeutic use in Th2-driven diseases such as allergy and cutaneous leishmaniasis.

Experimental

General experimental procedures: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-300 NMR spectrometer (^{1}H: 300 MHz; ^{13}C: 75 MHz). Reversed-phase chromatography was performed on RP-2 (70-230 mesh, Merck) and G-15-120 Sephadex (40-120 μm, Sigma). Eluates were monitored by TLC on silica 60 F_{254} (Merck) using *n*-butanol/acetic acid/water (BAW 8:1:1) and ethyl acetate/acetone/acetic acid/water (EAAW 30:3:1:1). TLC plates were visualized under UV light and with cerium sulfate.

Plant material: Flowers from *K. pinnata* were collected from specimens cultivated in the UFRJ campus (Rio de Janeiro, Brazil). A voucher specimen (292.697) was identified by the botanist M. F. Freitas at the herbarium of the Rio de Janeiro Botanical Garden (Brazil).

Flower and leaf extracts: Fresh flowers (2.52 kg) and fresh leaves (6.76 kg) were ground and extracted with distilled water at 20% w/v for 30 min at 50ºC. The yields of lyophilized flower and leaf extracts were 3.82% (90.2 g) and 2.16% (146.0 g), respectively, from fresh starting material (w/w).

Flavonoid isolation from KP flower extract: Dried flower extract was re-suspended in distilled water (2.1 L) and precipitated with EtOH (1:1). The soluble fraction (550 mL; corresponding to 76.9 g) was partitioned with ethyl acetate (1 x 550 mL; 2 x 225 mL), affording KP-FAcOEt (5.58 g; 5.8%, w/w of the dried extract). An aliquot of KP-FAcOEt (5.00 g) was re-suspended in distilled water (5 mL) and purified on a RP-2 column (8.0 x 3.9 cm; H_2O/MeOH gradient) affording 4 fractions. The flavonoid fraction KP-FAcOEt-02, eluted with 3:7 MeOH/H_2O (450 mL; 1.0562 g) was submitted to a RP-2 column (29.0 x 2.2 cm; H_2O/MeOH gradient), yielding **1** (quercetin) (10:0 MeOH/H_2O; 230 mL; 1.6 mg; R_f 0.84 - EAAW 30:3:1:1); ^{1}H and ^{13}C NMR (CD_3OD), as previously reported [12], and a fraction (MeOH/H_2O 3:7; 400 mL; 179.9 mg) containing 2 flavonoids. This fraction was purified on a Sephadex LH-20 column (32.0 x 2.5 cm; EtOH) affording **2** {quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside} (35.5 mg; R_f 0.55 - BAW, 8:1:1); ^{1}H and ^{13}C NMR (CD_3OD), as previously reported [10], and **3** (miquelianin) (46.5 mg; R_f 0.28 - BAW 8:1:1); ^{1}H and ^{13}C NMR (CD_3OD) as reported by Agrawal [12]. The second flavonoid fraction, KP-FAcOEt-03 (MeOH/H_2O 5:5 and 7:3; 500 mL; 1.338 g) was chromatographed on a RP-2 column (29.0 x 2.2 cm; H_2O/MeOH gradient) followed by a Sephadex LH-20 column (32.0 x 2.5 cm; EtOH) yielding an enriched flavonoid fraction (58.4 mg) that was purified on a RP-18 column (31.0 x 0.8 cm; H_2O/EtOH gradient) to afford **4** (isoquercitrin) (12.7 mg; R_f 0.35 - EAAW 30:3:1:1); ^{1}H and ^{13}C NMR (CD_3OD), as previously reported [12], and **5** (quercitrin) (19.2 mg; R_f 0.83 - BAW 8:1:1); ^{1}H and ^{13}C NMR (CD_3OD), as previously reported [9].

High performance liquid chromatography, quantitation and purity control of flavonoids: HPLC-DAD analysis was performed in triplicate (100 μg for each sample) on a HP 1100L instrument with a Diode Array Detector and managed by a HP 9000 workstation (Hewlett Packard, Palo Alto, CA, USA). A RP-18 reverse-column (5 μm, 250 mm x 4.60 mm, Luna, Phenomenex, USA) maintained at 26 ºC was used, as previously reported [11].

Animals: BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Animals were used at 4-6 weeks of age according to experimental protocols approved by the Animal Use Committee of the Federal University of Rio de Janeiro (CEUA, UFRJ, and Brazil).

T cell proliferation assay: The inguinal and popliteal lymph nodes were isolated from mice and single cell suspensions were prepared as reported [6a]. Viable Trypan Blue-negative cells (4 x 10^6 cells/mL) were stimulated with 5 μg/mL of the T cell mitogen concanavalin A (Sigma Chem. Co, USA) in either the absence or presence of the plant extracts (25 μg/mL, 50 μg/mL and 100 μg/mL) or flavonoids (1 μg/mL, 10 μg/mL and 100 μg/mL). Cyclosporin A (Sigma Chem. Co) was used as positive control. The proliferative response was evaluated through the incorporation of ^{3}H-thymidine (0.5 μ Ci/ well) added in the last 18 h of culture. Cells were then harvested using a Dot-Blot device [18b] and the radioactivity measured in a β-counter. The IC_{50} values were calculated by non-linear regression analysis.

Cytotoxicity: Macrophages were harvested from the peritoneal cavity of BALB/c mice and allowed to adhere at 1.5 x 10^6 cells/well to 24-well culture plates for 2 h. The isolated macrophages were cultured (24 h; 37°C) with varying concentrations of the samples. The LDH content was determined colorimetrically, as reported [9].

Cytokine production: Lymph node cells were prepared as for proliferation, and 0.5 mL aliquots of the samples were seeded in 24-well culture plates. Cells were cultured in either the absence or presence of flavonoids (25 μg/mL) and stimulated with concanavalin A (5 μg/mL) for 48 h. The levels of IL–2 and IL–4 were determined as reported previously [6a].

Statistical analysis: The statistical significance between control and treated samples was calculated by one-way analysis of variance followed by a Tukey post-test (significant when $p<0.05$).

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