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The antileishmanial activity assessment of unusual flavonoids from *Kalanchoe pinnata*

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Abstract

The importance of flavonoids for the antileishmanial activity of *Kalanchoe pinnata* was previously demonstrated by the isolation of quercitrin, a potent antileishmanial flavonoid. In the present study, the aqueous leaf extract from the medicinal plant *K. pinnata* (Crassulaceae) afforded a kaempferol di-glycoside, named kapinnatoside, identified as kaempferol 3-*O*-α-L-arabinopyranosyl (1 → 2) α-L-rhamnopyranoside (**1**). In addition, two unusual flavonol and flavone glycosides already reported, quercetin 3-*O*-α-L-arabinopyranosyl (1 → 2) α-L-rhamnopyranoside (**2**) and 4′,5-dihydroxy-3′,8-dimethoxyflavone 7-*O*-β-D-glucopyranoside (**3**), have been isolated. Their structures were determined via analyses of mono and bi-dimensional ^{1}H and ^{13}C NMR spectroscopic experiments and HR-MALDI mass spectra. Because of its restricted occurrence and its abundance in *K. pinnata*, flavonoid (**2**) may be a chemical marker for this plant species of high therapeutic potential. The three flavonoids were tested separately against *Leishmania amazonenis* amastigotes in comparison with quercitrin, quercetin and afzelin. The quercetin aglycone – type structure, as well as a rhamnosyl unit linked at C-3, seem to be important for antileishmanial activity.
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Keywords: *Kalanchoe pinnata*; Crassulaceae; Leishmaniasis; NMR; Flavonoids; Kaempferol 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside

1. Introduction

Kalanchoe pinnata (Lamarck) Persoon (=*Bryophyllum pinnatum*) is a perennial medicinal herb, popularly used in Brazil and other parts of the world to treat various inflammatory diseases (Rossi-Bergmann et al., 1994). Previous studies on the chemical composition of *K. pinnata* showed that bufadienolides, terpenoids and flavonoids are the main secondary metabolites of this species (Yamagishi et al., 1989; Costa et al., 1995). Our interest in *K. pinnata* is justified by its significant immunosuppressive effects, as well as its ability to protect against progressive infection with *Leishmania amazonensis* (Rossi-Bergmann et al., 1994; Da Silva et al., 1995).

The leishmaniases are a complex of diseases caused by different species of the protozoan parasite *Leishmania* and are a major public health problem in many developing countries, where 350 million people live at risk of infection (WHO, 2005). There is no approved vaccine for clinical use. Despite a few research achievements, first-line chemotherapy is still based on pentavalent antimonials, developed more than 50 years ago, which are toxic and prone to drug resistance (Croft and Coombs, 2003).

Recently, several natural products with antileishmanial activity, including naphthoquinones, lignans, neolignans, alkaloids and triterpenoids have been reported (Chan-

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Bacab and Peña-Rodriguez, 2001). However, there have been few studies on the antileishmanial activity of the flavonoid class of natural polyphenols. These few studies include that of luteolin, a common flavonoid in the human diet, which was recently described as a promising antileishmanial drug (Mittra et al., 2000). Proanthocyanidins also show antileishmanial activity, as well as modulatory effects on nitric oxide and tumor necrosis factor-alpha release in RAW 264.7 cells (Kolodziej et al., 2001), and a methoxychalcone isolated from inflorescences of *Piper aduncum* (Piperaceae) reportedly has significant antileishmanial activity as well (Torres-Santos et al., 1999). Quercitrin, previously isolated from an active flavonoid fraction of *K. pinnata* by our group, was an additional potent antileishmanial compound, with a low toxicity profile (Muzitano et al., 2006). Herein, we describe a new flavonoid diglycoside and two other polar flavonoids from *K. pinnata* as well as their *in vitro* antileishmanial activity in comparison with three analogs: quercetin, quercitrin, and afzelin.

2. Results and discussion

A *K. pinnata* aqueous extract was partitioned with dichloromethane under acidic (F1) and alkaline conditions (F2), then with ethyl acetate (F3). Fractionation of F3 using reversed-phase chromatography, followed by purification on G-15-120 Sephadex, afforded compound **2** as the major component of the fraction, and small amounts of **1** and **3**.

Compound **1** was isolated as an amorphous yellow powder and shown to be a flavonoid from its TLC visualization under UV light and cerium sulfate development. The molecular formula $C_{26}H_{28}O_{14}$ was deduced from the pseudomolecular ion at *m/z* 587.1358 $[M + Na]^+$(calculated for $C_{26}H_{28}O_{14}Na$, 587.1376) on the HR-MALDI mass spectrum. H^1 and 2-D NMR spectroscopic experiments were also performed using DMSO-d_6 and CD_3OD as solvents; the spectra in CD_3OD were used to measure the coupling constants of the carbohydrate hydrogens, because their signals were overlapped by water present in DMSO-d_6 spectra. The ^{13}C NMR spectroscopic experiments employed DMSO-d_6 as solvent.

The ^1H NMR (CD_3OD) spectra showed the presence of a kaempferol aglycone, as characterized by two doublet signals at δ 6.94 and 7.78, assigned to H-3′,5′ and H-2′,6′, respectively, and two doublet resonances at δ 6.20 (H-6) and 6.38 (H-8) (Table 1). The presence of a rhamnopyranosyl unit was suggested by a characteristic methyl doublet at δ 0.98 ($J = 6.17$ Hz) and a broad singlet corresponding to the anomeric proton at δ 5.47. Each carbohydrate spin system was assigned by analyses of ^1H-^1H COSY, HMQC and HMBC NMR spectroscopic experiments. An HMBC ^1H-^{13}C (DMSO-d_6) long-range correlation was observed between H-1″ (δ 5.34) and C-3 (δ 135.0), in agreement with the *O*-rhamnopyranosyl unit at C-3.

Five additional ^{13}C signals were observed at δ 104.3, 72.4, 71.2, 68.5 and 66.6 (DMSO-d_6), indicating the presence of a second carbohydrate unit which was identified as an arabinopyranosyl group from analysis of the ^1H–^1H coupling constants measured in the CD_3OD spectrum and by comparison with ^{13}C NMR spectroscopic data in the literature (Flamini et al., 2002). The large coupling constant (7.15 Hz) corresponding to the anomeric H-1‴ (δ 4.25) indicated a di-axial relation with H-2‴, consistent with the presence of an α-arabinopyranose moiety (Fig. 1 and Table 1). From the NMR spectroscopic data, the α-arabinopyranosyl moiety was inferred to be linked at the 2″-position of 3-*O*-rhamnopyranosyl unit (δ 81.2). This substitution pattern agreed with the deshielding effect observed for the C-2″ signal (+10 ppm) when compared to the C-2 signal of an unsubstituted 3-*O*-rhamnosyl unit (Slowing et al., 1994). From these data, compound 1 was concluded to be kaempferol 3-*O*-α-L-arabinopyranosyl (1 → 2) α-L-rhamnopyranoside for which we propose the name of kapinnatoside.

Flavonoid **2** was identified by analyses of ^1H, ^{13}C, COSY, HMQC, HMBC, NOESY and 1D-TOCSY NMR spectra as quercetin 3-*O*-α-L-arabinopyranosyl (1 → 2) α-L-rhamnopyranoside, whose complete ^1H and ^{13}C spectroscopic data and assignments are now reported for the first time (Tables 1 and 2). The molecular formula $C_{26}H_{28}O_{15}$ was inferred from the pseudomolecular ion at *m/z* 603.1309 $[M + Na]^+$ (calculated for $C_{26}H_{28}O_{15}Na$, 603.1326) in the HR-MALDI mass spectrum. This compound was previously isolated from *K. pinnata*, but only preliminary ^1H NMR data were available (Ichikawa et al., 1986). The comparison of our ^1H NMR spectroscopic data with those described by these authors shows a substantial difference between the H-1‴ anomeric coupling constants (CD_3OD). In our study, the measured coupling constant of H-1‴ (δ 4.20) is $J = 7.10$ Hz, while the coupling constant reported previously in the same solvent is $J = 4.00$ Hz (Ichikawa et al., 1986). Our data clearly indicate the di-axial relationship between H-1‴ and H-2‴ as expected for an α-arabinopyranosyl moiety; these data are in agreement with other literature reports (Flamini et al., 2002; Mabry et al., 1970). This quercetin di-glycoside (**2**) is an uncommon molecule not reported to date in other plant species, except for *Alphitonia philippinensis* (Rhamnaceae) (Jou et al., 2004).

K. pinnata has a high phytotherapeutic potential, as shown by its anti-inflammatory (Pal and Chaudhuri, 1990), anti-ulcer (Pal and Chaudhuri, 1991), hepatoprotective (Yadav and Dixit, 2003), antileishmania (Da Silva et al., 1995), immunomodulatory activities (Rossi-Bergmann et al., 1994) and tocolytic effectiveness (Plangger et al., 2006). Considering the requirement for quality control of phytomedicines, flavonoid **2**, which shows a restricted occurrence in nature, might therefore be considered as a chemical marker for this plant species.

Compound **3** was identified by analyses of the ^1H, ^{13}C, COSY, HMQC, HMBC, NOESY and NOE-sel NMR

Table 1
^{1}H NMR spectroscopic data for compounds **1**–**3**

	1		**2**		**3**
	δ_H [mult., *J* (Hz)]		δ_H [mult., *J* (Hz)]		δ_H [mult., *J* (Hz)]
	DMSO-d_6	CD_3OD	DMSO-d_6	CD_3OD	DMSO-d_6
Aglycone					
2	–	–	–	–	–
3	–	–	–	–	6.87 *s*
4	–	–	–	–	–
5	–	–	–	–	–
6	6.14 *s*	6.20 *d* (1.87)	6.16 *br s*	6.19 *d* (2.00)	6.59 *s*
7	–	–	–	–	–
8	6.34 *s*	6.38 *d* (1.87)	6.37 *br s*	6.37 *d* (2.00)	–
9	–	–	–	–	–
10	–	–	–	–	–
1′	–	–	–	–	–
2′	7.72 *d* (8.07)	7.78 *d* (8.68)	7.33 *br s*	7.36 *d* (2.07)	7.51 *br s*
3′	6.90 *d* (8.20)	6.94 *d* (8.70)	–	–	–
4′	–	–	–	–	–
5′	6.90 *d* (8.20)	6.94 *d* (8.70)	6.88 *d* (8.35)	6.93 *d* (8.32)	6.98 *d* (8.48)
6′	7.72 *d* (8.07)	7.78 *d* (8.68)	7.26 *br d* (8.35)	7.29 *dd* (2.07; 8.32)	7.55 *br d* (8.48)
3′- Me	–	–	–	–	3.95 *s*
8-Me	–	–	–	–	3.94 *s*
Rhamnosyl unit					
1″	5.34 *s*	5.47 *d* (1.37)	5.30 *br s*	5.37 *d* (0.93)	–
2″	*a*	4.20 *m*[b]	4.02 *m*[b]	4.19 *m*[b]	–
3″	*a*	3.82 *dd* (3.42; 9.66)	*a*	3.89 *dd* (3.70; 9.70)	–
4″	3.13 *dd* (9.29; 9.27)	3.32 dd[b] (9.62; 9.62)	*a*	3.35 *dd*[b] (9.70; 9.70)	–
5″	*a*	3.73 m[b]	*a*	3.87 *dq*[b] (9.70; 6.20)	–
6″	0.84 *d* (5.02)	0.98 *d* (6.17)	0.90 *d* (6.10)	1.10 *d* (6.20)	–
Arabinosyl unit					
1‴	4.10[a]	4.25 *d* (7.15)	4.09 *d* (6.37)	4.20 *d* (7.10)	–
2‴	3.39 *dd* (7.72; 8.10)	3.55 *dd* (7.38; 9.56)	*a*	3.54 *dd* (7.10; 9.28)	–
3‴	3.31 *m*	3.47 *dd* (3.25; 9.15)	*a*	3.47 *dd* (3.25; 9.28)	–
4‴	3.57 *br s*	3.73 *m*[b]	*a*	3.72 *m*[b]	–
5‴	3.48 *dd*[b] (11.80)	3.68 *m*[b]	*a*	3.65 *dd*[b] (12.45; 2.27)	–
	3.24 *br d*[b](11.80)	3.42 *br d*[b] (12.65)		3.37 *br d*[b] (12.45)	–
Glucosyl unit					
1″	–	–	–	–	5.02 *d* (6.55)
2″	–	–	–	–	3.33 *m*[b]
3″	–	–	–	–	3.17 *m*[b]
4″	–	–	–	–	3.33[a] *m*[b]
5″	–	–	–	–	3.43[a] *m*[b]
6″	–	–	–	–	3.43 *m*[b]; 3.71 *m*[b]

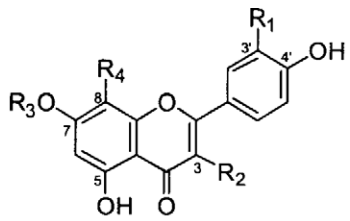
[a] Signals obscured by the water present in DMSO-d_6.
[b] Coupling constant not clearly defined or obscured by overlap.

spectra as being 4′,5-dihydroxy-3′,8-dimethoxyflavone 7-*O*-β-D-glucopyranoside, for which complete ^{1}H and ^{13}C spectroscopic data are described here for the first time. This is the second report of this flavonoid, which was first described in the foxtail grass *Setaria italica* (Poaceae), based only on preliminary ^{1}H NMR data of its hexaacetate derivative (Jain et al., 1991). Furthermore, this is the first report of a flavone glycoside from a *Kalanchoe* species.

The yield of flavonoid **2** corresponds to 0.049% of the dried extract, whereas flavonoids **1** and **3** were obtained in 0.0032% and 0.0025% yields, respectively.

Although a broad spectrum of biological activities has already been demonstrated for flavonoids, few studies have been devoted to the antileishmanial activity of this class of natural polyphenols. In order to evaluate their antileishmanial activity, flavonoids **1**–**3** were thus tested at three different concentrations on intracellular *L. amazonensis* amastigotes (Fig. 2). In addition, with the aim of establishing a structure–activity relationship, quercitrin (quercetin 3-*O*-α-L-rhamnopyranoside), quercetin and afzelin (kaempferol 3-*O*-α-L-rhamnopyranoside) were tested in the same experiment. The commercial drug Pentostam® was used as positive control ($IC_{50} \cong 20$ μg/mL). Results are shown in Fig. 2.

Quercetin 3-*O*-α-L-arabinopyranosyl (1 → 2) α-L-rhamnopyranoside (flavonoid **2**) exhibited the highest antileishmanial activity, $IC_{50} \cong 45$ μg/mL (78 μM), when compared with flavonoid **1**, $IC_{50} > 100$ μg/mL (>177 μM),



Flavonoids	R_1	R_2	R_3	R_4
1	H	*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranose	H	H
2	OH	*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranose	H	H
3	OMe	H	β-D-glucopyranose	OMe
quercitrin	OH	α-L-rhamnopyranose	H	H

Fig. 1. Kaempferol 3-*O*-α-L-arabinopyranosyl (1 → 2) α-L-rhamnopyranoside (**1**), quercetin 3-*O*-α-L-arabinopyranosyl (1 → 2) α-L-rhamnopyranoside (**2**), 4′,5-dihydroxy-3′,8-dimethoxyflavone 7-*O*-β-D-glucopyranoside (**3**) and quercitrin.

and flavonoid **3**, $IC_{50} > 100$ μg/mL (>203 μM). This difference can be seen clearly at 100 μg/mL, where flavonoid **2** inhibited amastigote growth by 60.5%, while flavonoids **1** and **3** inhibited by only 39.2% and 34.9%, respectively.

Considering all flavonoids tested, the most active ones, quercitrin, $IC_{50} \cong 8$ μg/mL (18 μM), and flavonoid **2**, have a quercetin aglycone, suggesting the importance of this structural feature for antileishmanial activity. This was confirmed further by the difference in activity between quercitrin and afzelin, $IC_{50} \cong 70$ μg/mL (162 μM), and between flavonoids **1** and **2**. This is also the first time that antileishmanial activity of afzelin is reported.

When comparing flavonoid **2** and quercitrin, both contain a quercetin aglycone. However, the difference in their activity profile is related to the presence of the arabinosyl unit linked at the inner rhamnosyl unit in flavonoid **2**. Taking into account the known rhamnose affinity of macrophage membranes, as recently demonstrated by Bonzue et al. (2005), the more facilitated interaction of the rhamnose moiety in quercitrin can be claimed to be, at least partially, implicated in the higher activity observed for this flavonoid. Interaction between the rhamnose moiety and the macrophage might also explain the differences in activity observed between quercitrin and quercetin, $IC_{50} > 100$ μg/mL (>331 μM).

3. Concluding remarks

Further investigation, including analyses of other analogs, should be carried out to obtain a better understanding of other aspects of flavonoid structure–activity relationships. It is also necessary to investigate the mechanism of action of such flavonoids in depth.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured on a Perkin–Elmer 243 B polarimeter using a sodium lamp (589 nm). ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-300 NMR spectrometer (^1H: 300 MHz; ^{13}C: 75 MHz) and on a Bruker DRX-400 NMR spectrometer (^1H: 400 MHz;^{13}C: 100 MHz). MS analyses were performed on a Biflex III (Bruker Daltonics, Billerica, MA, USA) in reflectron mode, IS1: 19 kV, IS2: 16.5 kV, reflector: 20 kV. Matrix: α-cyano-4-hydroxycinnamic (HCCA, Aldrich, Milwaukee, WI). For MS analysis, the sample was diluted in H_2O/MeOH (1:1, 1 μg/mL). An aliquot of 0.5 μL of sample solution, followed by 0.5 μL of matrix, was spotted on the target. MALDI spectra were internally calibrated using matrix peaks. Reversed-phase HPLC was performed on RP-2 (70–230 mesh, Merck) or RP-18 silanized silica (40–63 μM, Merck) and size-exclusion chromatography on G-15-120 Sephadex (40–120 μ, Sigma). Eluates were monitored by thin-layer chromatography (TLC) on silica 60 F_{254} (Merck) using *n*BuOH/AcOH/H_2O (BAW 8:1:1), visualized under UV light and revealed with cerium sulfate solution.

4.2. Plant material

K. pinnata was collected on the UFRJ campus out of blooming season (Ilha do Fundão, Rio de Janeiro). A voucher specimen (292.697) was deposited at the herbarium of the Rio de Janeiro Botanical Garden (Brazil) after identification by its staff.

Table 2
^{13}C NMR spectroscopic data for compounds **1**–**3** in DMSO-d_6

	1	2	3
Aglycone			
2	157.8	157.0	165.3
3	135.0	134.9	104.3
4	178.3	178.3	183.4
5	161.9	157.5	157.2
6	100.1	99.3	99.8
7	161.9	165.0	157.1
8	95.0	94.2	130.2
9	157.5	161.8	150.1
10	106.8	104.4	106.1
1′	121.3	121.2	122.6
2′	131.4	116.2	110.9
3′	116.4	145.8	149.2
4′	160.8	149.2	152.0
5′	116.4	116.0	117.1
6′	131.4	121.0	122.6
3′-OMe	–	–	56.2
8-OMe	–	–	62.5
Rhamnosyl unit			
1″	101.6	102.4	–
2″	81.2	81.1	–
3″	71.8	70.8	–
4″	73.1	73.0	–
5″	70.9	70.9	–
6″	18.1	17.8	–
Arabinosyl unit			
1‴	104.3	106.9	–
2‴	72.4	71.6	–
3‴	71.2	72.3	–
4‴	68.5	68.3	–
5‴	66.6	66.3	–
Glucosyl unit			
1″	–	–	101.3
2″	–	–	74.2
3″	–	–	77.5
4″	–	–	70.7
5″	–	–	78.1
6″	–	–	61.7

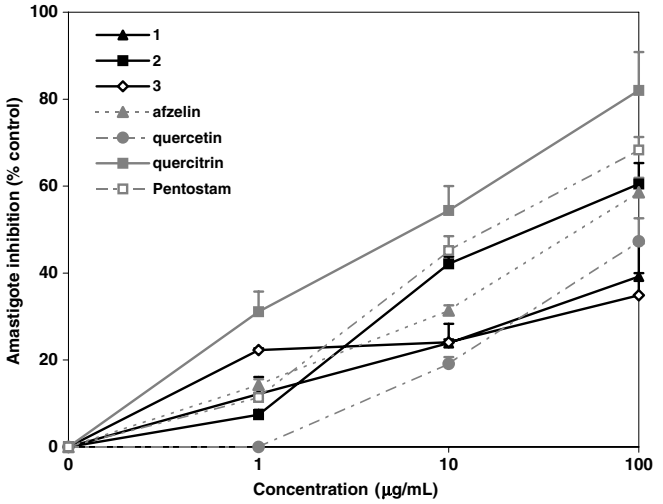


Fig. 2. Inhibition of *L. amazonensis* amastigote growth by quercitrin, afzelin, quercetin, kaempferol 3-*O*-α-L-arabinopyranosyl (1 → 2) α-L-rhamnopyranoside – flavonoid **1**, quercetin 3-*O*-α-L-arabinopyranosyl (1 → 2) α-L-rhamnopyranoside – flavonoid **2** and 4′,5-dihydroxy-3′,8-dimethoxyflavone 7-*O*-β-D-glucopyranoside – flavonoid **3**. Pentostam® was used as positive control. Arithmetic means ± standard deviation ($n = 3$). Untreated, infected macrophages (negative control) = 29398 UF and untreated, uninfected macrophages (positive control) = 4427.6 UF, where UF means units of fluorescence.

4.3. Extraction and isolation

Fresh leaves (6.76 kg) were triturated and extracted with distilled H_2O at 20% (w/v) for 30 min at 50 °C. The extract was concentrated to 1/5 of its initial volume at the same temperature and partitioned with CH_2Cl_2 at pH 2, with concd. HCl, and then at pH 11, with 10 N NaOH, to obtain *F*1 (423.1 mg) and *F*2 (113.0 mg), respectively. The residual aqueous phase was neutralized and partitioned using EtOAc, to obtain *F*3 (1.110 g). An aliquot of *F*3 (1.067 g) was re-suspended in distilled H_2O (5 mL) and subjected to RP-2 cc (29.0 × 2.2 cm; H_2O/MeOH gradient), yielding 3 fractions: F3-1 eluted with 0:10 MeOH/H_2O until 3:7 MeOH/H_2O (500 mL; 354.9 mg), F3-2 eluted with 3:7 MeOH/H_2O until 5:5 MeOH/H_2O (300 mL; 496.7 mg) and F3-3 eluted with 5:5 MeOH/H_2O until 10:0 MeOH/H_2O (600 mL; 215.0 mg). The only flavonoid fraction, F3-2, was purified on an RP-18 column (32.0 × 2.5 cm; H_2O/EtOH gradient), yielding two flavonoid fractions. A small amount of a yellow solid appeared during solvent evaporation of the second flavonoid fraction (eluted with 3:7 EtOH/H_2O – 10:0 EtOH/H_2O, elution volume: 320 mL). This solid material was separated by centrifugation and corresponded to compound **3**: 4.0 mg; R_f 0.70. After separation of **3**, the mother-liquor (265.2 mg), enriched in one compound, was subjected Sephadex G-15 cc (31.0 × 0.8 cm; H_2O) affording compound **2**: 78.5 mg; R_f 0.55; and a small amount of compound **1**: 5.2 mg; R_f 0.61.

4.3.1. Kaempferol 3-*O*-α-L-arabinopyranosyl (1 → 2) α-L-rhamnopyranoside (*1*)

Amorphous yellow powder; $[\alpha]_D^{25} = -94$ (MeOH; c 1.0); for NMR data, see Tables 1 and 2; HR-MALDI *m*/*z* 587.1358 $[M + Na]^+$ (calculated for $C_{26}H_{28}O_{14}Na$, 587.1376).

4.3.2. Quercetin 3-*O*-α-L-arabinopyranosyl (1 → 2) α-L-rhamnopyranoside (*2*)

Amorphous yellow powder; $[\alpha]_D^{25} = -53$ (MeOH; c 1.0); for NMR data, see Tables 1 and 2; HR-MALDI *m*/*z* 603.1309 $[M + Na]^+$ (calculated for $C_{26}H_{28}O_{15}Na$, 603.1326).

4.3.3. 4′,5-dihydroxy-3′,8-dimethoxyflavone 7-*O*-β-D-glucopyranoside (*3*)

Amorphous yellow powder; $[\alpha]_D^{25} = -380$ (MeOH; c 1.0); for NMR data, see Tables 1 and 2.

4.4. Biological assay

Parasite *L. amazonensis* (MHOM/BR/75/Josefa strain) promastigotes transfected with green fluorescent protein (*L. amazonensis*-GFP promastigotes) were used. They were routinely isolated from mouse lesions and maintained as promastigotes in Dulbecco modified Eagle medium containing 10% heat-inactivated fetal calf serum and 150 μg Geneticin/mL at 26 °C.

4.4.1. Antileishmanial activity

For antiamastigote activity, mouse peritoneal macrophages were plated in triplicate in 24-well culture microplates at 2×10^6 cells/well in 0.4 mL complete medium. After 1 h at 37 °C and 5% CO_2, the non-adherent cells were removed and the cells were infected with 10^7 promastigotes/well for 4 h at 34 °C. Free parasites were washed away with pre-warmed saline and the infected cells were cultured for an additional 72 h in 0.5 mL of medium, in the presence or absence of plant material at different concentrations. After the culture supernatants were collected, the cells were transferred using distilled H_2O (200 μl) of to black microplates, and the fluorescence was read in a plate reader fluorimeter (Fluoroskan; LabSystems) at 435-nm excitation and 538-nm emission.

Concentrations were plotted (Fig. 2) as μg/mL because Pentostam, the positive control, is a complex polymeric antimonial (Berman and Grogl, 1988) and its molecular weight has not been determined (Croft and Yardley, 2002). In order to allow direct comparison, flavonoid IC_{50} values are reported in the text as μg/mL and μM.

4.4.2. Chemicals

Sodium stibogluconate (Pentostam), Glaxo Wellcome Research and Development (GW387208A0044X60). Quercitrin, quercetin and afzelin, were authentic samples isolated previously in our laboratory.

This research has complied with all relevant federal guidelines and institutional policies related to the use of animal models for research purposes.

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References

Berman, J.D., Grogl, M., 1988. *Leishmania mexicana*: chemistry and biochemistry of sodium stibogluconate (Pentostam). Exp. Parasitol. 67, 96–103.

Bonzue, J.A., Parthasarathy, N., Phillips, L.R., Cote, C.K., Fellows, P.F., Mendelson, I., Shafferman, A., Friedlander, A.M., 2005. Construction of a rhamnose mutation in *Bacillus anthracis* affects adherence to macrophages but not virulence in guinea pigs. Microb. Pathog. 38, 1–12.

Chan-Bacab, M.J., Peña-Rodriguez, L.M., 2001. Plant natural products with leishmanicidal activity. Nat. Prod. Rep. 18, 674–688.

Costa, S.S., Jossang, A., Bodo, B., 1995. In: Allorge-Boiteau (Ed.), *Kalanchoe* (Crassulacées) de Madagascar, Systématique, Écophysiologie et Phytochimie, vol. 1, Paris, p. 219.

Croft, S.L., Coombs, G.H., 2003. Leishmaniasis – current chemotherapy and recent advances in the search for novel drugs. Trends Parasitol. 19, 502–508.

Croft, S.L., Yardley, V., 2002. Chemotherapy of leishmaniasis. Curr. Pharm. Des. 8, 319–342.

Da Silva, S.A.G., Costa, S.S., Mendonça, S.C.F., Silva, E.M., Moraes, V.L.G., Rossi-Bergmann, B., 1995. Therapeutic effect of oral *Kalanchoe pinnata* leaf extract in murine leishmaniasis. Acta Trop. 60, 201–205.

Flamini, G., Pardini, M., Morelli, I., Ertugrul, K., Dural, H., Bagci, Y., Kargioglu, M., 2002. Flavonoid glycosides from *Centaurea pseudoscabiosa* subsp. *pseudoscabiosa* from Turkey. Phytochemistry 61, 433–437.

Ichikawa, M., Ogura, M., Iijima, T., 1986. Antiallergic flavone glycoside from *Kalanchoe pinnatum*. Patent 61, 118, 396 [86, 118, 319] (Cl. CO7H17/07), Apl 84/240, 282, 14 nov 1984; 4p-Chemical abstracts, 105, 178423q, 1986.

Jain, N., Ahmad, M., Kamil, M., Ilyas, M., 1991. 8,3′-dimethoxy-5,4′-dihydroxyflavone 7-glucoside from *Setaria italica*. Phytochemistry 30, 1345–1347.

Jou, S.J., Chen, C.H., Guh, J.H., Lee, C.N., Lee, S.S., 2004. Flavonol glycosides and cytotoxic triterpenoids from *Alphitonia philippinensis*. J. Chin. Chem. Soc. 51, 827–834.

Kolodziej, H., Kayser, O., Kiderlen, A.F., Ito, H., Hatano, T., Yoshida, T., Foo, L.Y., 2001. Proanthocyanidins and related compounds: Antileishmanial activity and modulatory effects on nitric oxide and tumor necrosis factor-alpha-release in the murine macrophage-like cell line RAW 264.7. Biol. Pharm. Bull. 24, 1016–1021.

Mabry, T.J., Markham, K.R., Thomas, M.B., 1970. The Systematic Identification of Flavonoids. Springer, New York.

Mittra, B., Saha, A., Chowdhury, A.R., Pal, C., Mandal, S., Mukhopadhyay, S., Bandyopadhyay, S., Majumder, H.K., 2000. Luteolin, an abundant dietary component, is a potent antileishmanial agent that acts by inducing topoisomerase II-mediated kinetoplast DNA cleavage leading to apoptosis. Mol. Med. 6, 527–541.

Muzitano, M.F., Cruz, E.A., Almeida, A.P., Silva, S.A.G., Kaiser, C.R., Guette, C., Rossi-Bergmann, B., Costa, S.S., 2006. Quercitrin: an antileishmanial flavonoid glycoside from *Kalanchoe pinnata*. Planta Med. 72, 81–83.

Pal, S., Chaudhuri, A.K.N., 1990. Anti-inflammatory action of *Bryophyllum pinnatum* leaf extract. Fitoterapia 6, 527–533.

Pal, S., Chaudhuri, A.K.N., 1991. Studies on the anti-ulcer activity of a *Bryophyllum pinnatum* leaf extract in experimental animals. J. Ethnopharmacol. 33, 97–102.

Plangger, N., Rist, L., Zimmermann, R., von Mandach, U., 2006. Intravenous tocolysis with *Bryophyllum pinnatum* is better tolerated than beta-agonist application. Eur. J. Obstet. Gynecol. Reprod. Biol. 124, 168–172.

Rossi-Bergmann, B., Costa, S.S., Borges, M.B.S., Da Silva, S.A.G., Noleto, G.R., Souza, M.L.M., Moraes, V.L.G., 1994. Immunosuppressive effect of the aqueous extract of *Kalanchoe pinnata* in mice. Phytother. Res. 8, 399–402.

Slowing, K., Sollhuber, M., Carretero, E., Villar, A., 1994. Flavonoid glycosides from *Eugenia jambos*. Phytochemistry 37, 255–258.

Torres-Santos, E.C., Moreira, D.L., Kaplan, M.A.C., Meirelles, M.N., Rosi-Bergmann, B., 1999. Selective effect of 2′,6′-dihydroxy-4′-methoxychalcone isolated from *Piper aduncum* on *Leishmania amazonensis*. Antimicrob. Agents Chemother. 43, 1234–1241.

World Health Organization (WHO), Division of Control of Tropical Diseases. <http://www.who.int/tdr/diseases/leish/diseaseinfo.htm> (accessed 00.11.05).

Yadav, N.P., Dixit, V.K., 2003. Hepatoprotective activity of leaves of *Kalanchoe pinnata* Pers. J. Ethnopharmacol. 86, 197–202.

Yamagishi, T., Haruna, M., Yan, X-Z., Chang, J.J., Lee, K.H., 1989. Antitumor agents, 110[1,2] Bryophyllin B, a novel potent cytotoxic bufadienolide from *Bryophyllum pinnatum*. J. Nat. Prod. 52, 1071–1079.