

CHEMICAL CONSTITUENTS FROM *Bakeridesia pickelii* MONTEIRO (MALVACEAE) AND THE RELAXANT ACTIVITY OF KAEMPFEROL-3-O- $\beta$ -D-(6''-E-p-COUMAROYL) GLUCOPYRANOSIDE ON GUINEA-PIG ILEUM

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The phytochemical investigation of *Bakeridesia pickelii* Monteiro led to the isolation of seven compounds:  $\beta$ -sitosterol, a mixture of sitosteryl-3-O- $\beta$ -D-glucopyranoside and stigmasteryl-3-O- $\beta$ -D-glucopyranoside, vanillic acid, *p*-coumaric acid, quercetin 3-O- $\beta$ -D-glucopyranoside (isoquercitrin) and kaempferol-3-O- $\beta$ -D-(6''-E-p-coumaroyl) glucopyranoside (tiliroside), which was isolated as the major component. Their structures were elucidated on the basis of spectroscopic data such as IR,  $^1\text{H}$  and  $^{13}\text{C}$  NMR, including two-dimensional techniques. Tiliroside relaxed the guinea-pig ileum pre-contracted with KCl 40 mM ( $\text{EC}_{50} = 9.5 \pm 1.0 \times 10^{-5}$  M), acetylcholine  $10^{-6}$  M ( $\text{EC}_{50} = 2.3 \pm 0.9 \times 10^{-5}$  M) or histamine  $10^{-6}$  M ( $\text{EC}_{50} = 4.1 \pm 1.0 \times 10^{-5}$  M) in a concentration-dependent manner.

Keywords: malvaceae; *Bakeridesia pickelii*; relaxant activity.

## INTRODUCTION

The Malvaceae family comprises about 243 genera and 4225 species in the new phylogenetic circumscription<sup>1</sup>. Members of this family are widespread in many regions of the world and particularly in tropical regions, mainly in South America<sup>2</sup>. Many species of Malvaceae are known by their different uses in folk medicine such as diuretic, in treatment of rheumatism, gastrointestinal disorders<sup>3,4</sup>, snakebites<sup>5</sup> and asthma<sup>6</sup>. Antiinflammatory and antinociceptive effects have also been investigated<sup>7</sup>. Previous phytochemical investigations of Malvaceae species describe the isolation of fatty acids<sup>8-11</sup>, essential oils<sup>12</sup>, sesquiterpenoids of cadinene-type<sup>13</sup>, to which has been attributed an insecticide activity, sesquiterpenelactones<sup>14</sup>, triterpenes<sup>15</sup> and flavonoids<sup>16,17</sup>, among many others compounds. Based on the great variety of natural compounds found in species of the Malvaceae family as well as their pharmacological properties, we chose *Bakeridesia pickelii*, an endemic and unstudied species of the Brazilian Northeast to accomplish our study.

This work describes the first phytochemical investigation of *Bakeridesia pickelii* and the relaxant activity of kaempferol-3-O- $\beta$ -D-(6''-E-p-coumaroyl) glucopyranoside (tiliroside) on guinea-pig ileum.

## EXPERIMENTAL

### NMR and chromatographic procedures

NMR spectra ( $^1\text{H}$ ,  $^{13}\text{C}$ , HMQC, HMBC, COSY) were run on a Mercury Varian instrument operating at 200 MHz and 50 MHz for  $^1\text{H}$  and  $^{13}\text{C}$ , respectively. The NMR data were measured in  $\text{CDCl}_3$ ,  $\text{CD}_3\text{OD}$  and pyridine- $d_5$  and chemical shifts are expressed in ppm with reference to the solvent signal. Chromatography columns were performed on silica gel (Merck) and Sephadex LH-20 (Pharmacia, Uppsala-Sweden). The fractions were monitored by TLC analysis, using plates coated with silica gel 60 F<sub>254</sub> (Merck). The plates were

developed with iodine, visualized under UV light (244 and 366 nm), and spraying with 5%  $\text{FeCl}_3$  reagent (for detection of glycosyl flavonoids).

### Plant material

The aerial parts of *B. pickelii* were collected in May, 25, 2002 in the Pico do Jabre, municipality of Maturéia in State of Paraíba, and identified by Dr. M. de F. Agra. The voucher specimen *Agra et al. 5151* is deposited at the Herbarium Prof. Lauro Pires Xavier (JPB), Universidade Federal da Paraíba, Paraíba, Brazil.

### Extraction and isolation

Dried and ground aerial parts of *B. pickelii* (5400 g) were extracted with ethanol (3 x 10 L, 72 h each) and the crude extract was concentrated to dryness under reduced pressure to yield 190 g of a dark green residue. This material was partitioned with hexane, chloroform, ethyl acetate and butanol. The hexane extract (10 g) was chromatographed over silica gel 60 column (0.063-0.200 mm, Merck) using a gradient of hexane-EtOAc and EtOAc-MeOH. Fractions of 50 mL were collected. The fractions 33-52 were analysed by TLC ( $\text{SiO}_2$ , hexane-EtOAc 9:1), combined and recrystallized with  $\text{CHCl}_3$ :MeOH yielding  $\beta$ -sitosterol (**1**) (72 mg). Fractions 70-100 were combined after TLC analysis yielding a mixture of sitosteryl-3-O- $\beta$ -D-glucopyranoside (**2**) and stigmasteryl-3-O- $\beta$ -D-glucopyranoside (**3**) (104 mg). The chloroform extract (19 g) was chromatographed on silica gel 60 column (0.063-0.200 mm, Merck) with  $\text{CHCl}_3$ ,  $\text{CHCl}_3$ -EtOAc, EtOAc and EtOAc:MeOH. The fraction eluted with  $\text{CHCl}_3$ -EtOAc was also submitted to chromatography over silica gel 60 (0.063-0.200 mm, Merck), using hexane-EtOAc and EtOAc-MeOH as eluent. Fractions of 70 mL were collected and monitored by TLC. Fractions 01-85 combined and chromatographed over Sephadex LH-20 (Pharmacia, Uppsala-Sweden) and eluted with  $\text{CHCl}_3$ :MeOH (50:50, fractions of 20 mL). The fractions 23-24 and 28-34 were combined after observation on TLC ( $\text{SiO}_2$ ,  $\text{CHCl}_3$ :MeOH 7:3,  $\text{FeCl}_3$  5%) and recrystallized with MeOH, leading to the isolation

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of vanillic acid (**4**) (30 mg) and *p*-coumaric acid (**5**) (30 mg), respectively. Ethyl acetate extract (3 g) was applied to a Sephadex LH-20 column (Pharmacia, Uppsala-Sweden) with MeOH as eluent, where fractions of 20 mL were obtained. Fractions 07-11 and 12-25 were combined after analysis through TLC (SiO<sub>2</sub>, CHCl<sub>3</sub>:MeOH 1:1, FeCl<sub>3</sub> 5%) and finally recrystallized with MeOH, furnishing quercetin 3-O-β-D-glucopyranoside (isoquercitrin) (**6**) (40 mg) and kaempferol 3-O-β-D-(6''-E-*p*-coumaroyl) glucopyranoside (tiliroside) (**7**) (180 mg), respectively (Figure 1).

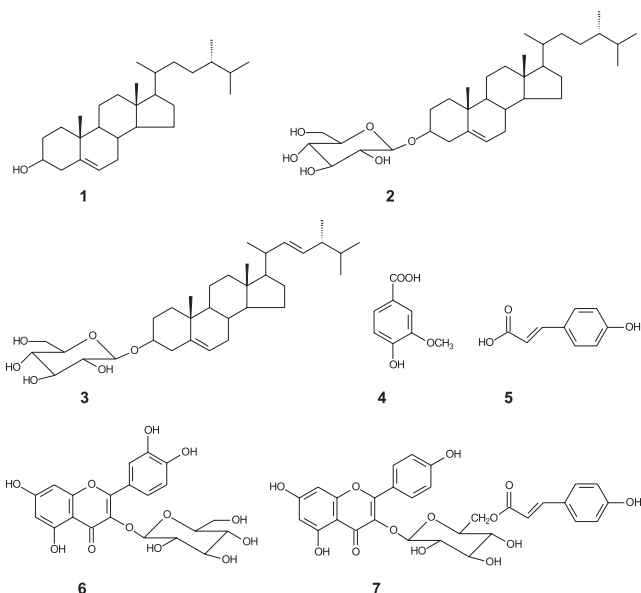


Figure 1. Chemical constituents isolated from *B. pickelii*

## Animals

Adult guinea-pigs (*Cavia porcellus*) of either sex weighing from 300 to 500 g were used in all experiments. The animals were submitted to the following conditions: 12 h light:12 h dark cycle, ventilation and temperature controlled (22 ± 1 °C) and were deprived of food for 18 h before the beginning of the experiments. The experimental procedure was approved by the Animal Experimentation Committee of the Universidade Federal da Paraíba.

## Reagents

NaHCO<sub>3</sub>, KCl and MgSO<sub>4</sub>·7H<sub>2</sub>O (Reagen); acetylcholine, CaCl<sub>2</sub>·H<sub>2</sub>O, NaCl, MgCl<sub>2</sub>·6H<sub>2</sub>O, NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, glucose (Merck) and histamine (Sigma-Aldrich).

## Tissue preparation

To perform *in vitro* studies, guinea-pig ileum was prepared according to Daniel *et al.*<sup>18</sup>. The guinea-pigs were killed by cervical dislocation, the abdomen opened and the ileum was immediately removed. Only the distal portions, with 3 cm length, were used. The tissues were placed in 6 mL isolated organ baths containing modified Krebs solution<sup>19</sup> with the following composition (mM): NaCl 117, KCl 4.7, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.3, NaH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25 and glucose 11, bubbled continuously with a 95% O<sub>2</sub> and 5% CO<sub>2</sub> gas mixture and maintained at 37 °C. The pH was adjusted to 7.4 with HCl 1 N. Tension changes were recorded through an isometric force transducer (7003) counterbalanced by 1 g loading, connected with a polygraph (Gemini 7070), both from Ugo Basile (Italy).

At the beginning of each experiment, the reactivity of the ileum was tested with KCl 40 mM. After washout with modified Krebs solution and following 30 min recovery period, contractions were evoked by adding KCl 40 mM, acetylcholine 10<sup>-6</sup> M or histamine 10<sup>-6</sup> M.

## Effect of tiliroside on tonic contractions induced by histamine, acetylcholine or KCl

After resting period, an isometric contraction was elicited in response to histamine 10<sup>-6</sup> M, acetylcholine 10<sup>-6</sup> M or KCl 40 mM, which provides an 80% of the possible maximum response for each agent (determined from preliminary experiments). Histamine, acetylcholine or KCl remained in contact with the preparation until a contraction plateau was reached (approximately 8 min). After that time the tissue was washed. After a period of 30 min, the process was repeated and on the contraction plateau, tiliroside was added cumulatively. Subsequent concentrations were added only when the response to the previous concentration became stable. Relaxation was expressed as a percentage of decrease of the maximum tension obtained by histamine, acetylcholine or KCl.

## Statistical analysis

Values were expressed as mean ± S.E.M. and "n" refers to the number of animals used in each set of experiments. Statistical analysis was performed using the GraphPad Prism® 3.03 software (GraphPad Software Inc., San Diego CA, 92121, USA). The EC<sub>50</sub> values were determined by non-linear regression from concentration-response curves<sup>20</sup>. Differences between the means were statistically compared using Student's "t" test or one-way ANOVA followed by Bonferroni's test, and a *p* < 0.05 was considered significant.

**Quercetin-3-O-β-D-glucopyranoside (isoquercitrin) (6):** IR (KBr)  $\bar{\nu}_{\max}$  3422, 3276, 2958, 2922, 1655, 1605, 1460, 1300, 1088; <sup>1</sup>H NMR [(200 MHz, CD<sub>3</sub>OD,  $\delta$  (ppm), *J* (Hz)): 7.71 (*d*, *J* = 1.8 Hz, H-2'), 7.58 (*dd*, *J* = 8.5 and 1.8 Hz, H-6'),  $\delta$  6.86 (*d*, *J* = 8.5 Hz, H-5'), 6.40 (*d*, *J* = 1.8 Hz, H-8), 6.21 (*d*, *J* = 1.8 Hz, H-6), 5.24 (*d*, *J* = 7.9 Hz, H-1''), 3.80-3.29 (H-2'', H-3'', H-4'', H-5'', H-6''). RMN <sup>13</sup>C [(50 MHz, CDCl<sub>3</sub>,  $\delta$  (ppm)): 179.48 (C-4), 166.02 (C-7), 163.09 (C-5), 159.00 (C-2), 158.48 (C-9), 149.86 (C-4'), 145.92 (C-3'), 135.60 (C-3), 123.19 (C-6'), 123.05 (C-1'), 117.52 (C-2'), 116.00 (C-5'), 105.70 (C-10), 104.25 (C-1''), 99.88 (C-6), 94.67 (C-8), 78.40 (C-3''), 78.10 (C-5''), 75.70 (C-2''), 71.18 (C-4''), 62.50 (C-6'').

**Kaempferol 3-O-β-D-(6''-E-*p*-coumaroyl) glucopyranoside (tiliroside) (7):** yellow powder (MeOH); IR (KBr)  $\bar{\nu}_{\max}$  3460, 2923, 1648, 1655, 1607, 1501, 1181, 1066, 980 cm<sup>-1</sup>; <sup>1</sup>H NMR [(200 MHz, CD<sub>3</sub>OD,  $\delta$  (ppm), *J* (Hz)): 7.96 (*d*, *J* = 8.9 Hz, H-2',6'), 7.38 (*d*, *J* = 15.6 Hz, H-β), 7.26 (*d*, *J* = 8.5 Hz, H-2'',6''), 6.79 (*d*, *J* = 8.9 Hz, H-3',5'), 6.75 (*d*, *J* = 8.5 Hz, H-3'',5''), 6.25 (*d*, *J* = 2.2 Hz, H-8), 6.10 (*d*, *J* = 2.2 Hz, H-6), 6.05 (*d*, *J* = 15.6 Hz, H-α), 5.24 (*d*, *J* = 7.6 Hz, H-1''), 4.31 (*dd*, Ha-6''), 4.19 (*dd*, Hb-6''), 3.54 (*m*, H-2'', 3'', 4'', 5''); [(50 MHz, CDCl<sub>3</sub>,  $\delta$  (ppm)): 179.32 (C-4), 168.82 (CO), 165.81 (C-7), 162.82 (C-5), 161.46 (C-4'), 161.12 (C-4''), 159.25 (C-2), 158.27 (C-9), 146.52 (C-β), 132.21 (C-2',6'), 131.15 (C-2'',6''), 127.02 (C-1''), 122.63 (C-1'), 116.74 (C-3'',5''), 115.98 (C-3',5'), 114.71 (C-α), 105.54 (C-10), 104.03 (C-1''), 99.92 (C-6), 94.85 (C-8), 77.96 (C-3''), 75.73 (C-2'',5''), 71.68 (C-4''), 64.37 (CH<sub>2</sub>O-6'').

## RESULTS AND DISCUSSION

The structural identifications of the compounds **1-6** were based on spectral analysis and also by comparison with literature data,

which allowed their assignments as  $\beta$ -sitosterol (**1**)<sup>21</sup>, a mixture of sitosteryl-3-*O*- $\beta$ -D-glucopyranoside (**2**)<sup>21</sup> and stigmasteryl-3-*O*- $\beta$ -D-glucopyranoside (**3**),<sup>1</sup> vanillic acid (**4**)<sup>22</sup>, *p*-coumaric acid (**5**)<sup>23</sup> and quercetin-3-*O*- $\beta$ -D-glucopyranoside (**6**) (isoquercitrin)<sup>24</sup>.

The IR spectrum of compound **7** showed a hydroxyl signal at 3460 cm<sup>-1</sup>, a conjugated carbonyl ester absorbance at 1685 cm<sup>-1</sup> and aromatic C=C stretching absorptions at 1607 and 1600 cm<sup>-1</sup>. The <sup>1</sup>H NMR spectrum revealed a set of signals of kaempferol, a *p*-coumaroyl group and a sugar unit. The identity of kaempferol was confirmed by two doublets at  $\delta$  6.10 and  $\delta$  6.25 ( $J = 2.5$  Hz) for H-6 and H-8 respectively and a pair of doublets at  $\delta$  7.96 and  $\delta$  6.79 ( $J = 8.9$  Hz) for H-2',6' and H-3',5', respectively, corresponding to an AA',BB' system. The *p*-coumaroyl group was characterized by the presence of A,A',B,B'-type aromatic proton signals at  $\delta$  7.26 (2H, *d*,  $J = 8.5$  Hz) and  $\delta$  6.75 (2H, *d*,  $J = 8.5$  Hz) and two-*trans*-olefinic hydrogen signals at 6.05 (1H, *d*,  $J = 15.6$  Hz) and 7.38 (1H, *d*,  $J = 15.6$  Hz). In the sugar region of the <sup>1</sup>H NMR spectrum were observed absorptions at  $\delta$  3.28-4.31 and also a doublet at  $\delta$  5.24 ( $J = 7.6$  Hz) related to the anomeric proton H-1'' of glucose. The diaxial coupling ( $J = 7.6$  Hz) between H-1'' and H-2'' suggested a  $\beta$ -configuration<sup>25</sup>.

The linkage of the glucose in **7** to the C<sub>3</sub> position of kaempferol was deduced on the basis of the HMBC spectrum analysis, which showed the cross-peak correlation between H-1'' ( $\delta$  5.24) and C-3 ( $\delta$  135.23). The higher field shift of C-6'' carbon signal of the glucose moiety compared with kaempferol-3-*O*- $\beta$ -D-glucopyranoside<sup>26</sup> indicated that the *p*-coumaroyl group might be attached to the C<sub>6</sub>-position of the glucose in **7**.

The HMQC spectrum of **7** revealed the correlation of appropriate hydrogen and carbon signals and the <sup>2</sup>*J* and <sup>3</sup>*J* correlations observed in the HMBC spectrum proved the unambiguous chemical shifts assignments of carbon atoms and substitution pattern of the molecule (Figure 2) Therefore, compound **7** was identified as kaempferol-3-*O*- $\beta$ -D-(6''-*E*-*p*-coumaroyl) glucopyranoside.

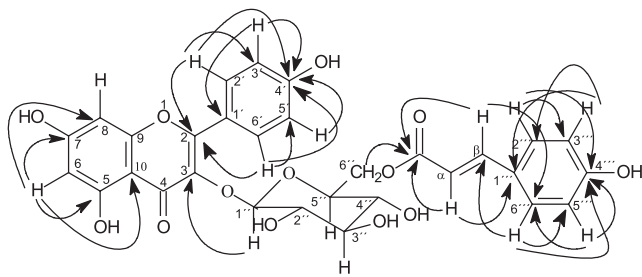


Figure 2. Main HMBC correlations of compound **7**

In the present study, we have investigated the effects of tiliroside on intestinal smooth muscle. The most important finding in this work is the demonstration for the first time that tiliroside exerts a non-selective relaxant action in guinea-pig ileum (data not shown).

Tiliroside, at 10<sup>-4</sup> M has not shown significant inhibitory effect on isotonic contractions induced by 10<sup>-6</sup> M histamine or acetylcholine in guinea-pig ileum. On the other hand, when the ileum was pre-contracted with acetylcholine, histamine or KCl, tiliroside induced a significant relaxant effect in a concentration-dependent manner, as shown in Figure 3.

Independently of the contraction being evoked by either pharmacomechanical (acetylcholine and histamine) or electromechanical (KCl) coupling, the maintenance of the tonic component involves activation of voltage-dependent Ca<sup>2+</sup> channels<sup>27</sup>. Therefore, we can postulate that tiliroside could be acting by the blockade of Ca<sup>2+</sup> influx through these channels to produce non-

selective relaxant effects, however further studies will be necessary to reinforce this hypothesis.

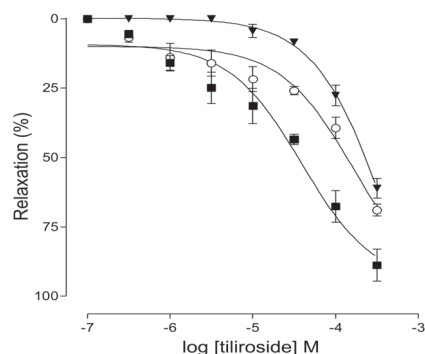


Figure 3. Effect of different concentrations of tiliroside on the histamine 10<sup>-6</sup> M (○), acetylcholine 10<sup>-6</sup> M (■) and KCl 40 mM (▼) induced tonic contractions in guinea-pig ileum (n = 6). The symbols and vertical bars represent means  $\pm$  S.E.M., respectively

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