IN VITRO EFFECT OF ISOCHAFTOSIDE ISOLATED FROM Syngonium podophyllum ON PIG KIDNEY Na⁺, K⁺-ATPase

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The present study aimed to investigate the in vitro effects of isoschaftoside isolated from Syngonium podophyllum on pig kidney Na⁺, K⁺-ATPase. The Na⁺, K⁺-ATPase activity was determined by colorimetric measurement of inorganic phosphate (Pi), resulting from ATP hydrolysis. Isoschaftoside significantly decreased the renal Na⁺, K⁺-ATPase activity at the highest concentration as well as at a lower concentration. Our work suggests that isoschaftoside is a promising compound for the treatment of hypertension.

Keywords: Syngonium podophyllum; Araceae; Isoschaftoside; enzyme inhibition; renal Na⁺, K⁺-ATPase.

INTRODUCTION

Kidneys are the main organs responsible for body fluid compartments homeostasis, notably for extracellular volume regulation and, consequently, regulation of circulatory parameters, such as, venous return, cardiac output and blood pressure. The maintenance of extracellular volume requires an accurate regulation of the amount of Na⁺ excreted and, reabsorbed at the tubular level. Therefore, the active Na⁺ transporter, Na⁺, K⁺-ATPase, is the target of a complex control system (neural and humoral factors) that modulates the renal excretion of Na⁺, and dysfunction of this system is one of the molecular bases of arterial hypertension of renal origin.¹

Na⁺, K⁺-ATPase is a P-type ATPase that is found in all eukaryotic cells, including the nephron cells, that are principally in the cortex region. This ATPase uses the chemical energy from ATP hydrolysis to transport Na⁺ outside the cell against its electrochemical gradient and transport K⁺ into cytoplasm. Thereby, maintaining low concentrations of Na⁺ and high concentrations of K⁺ inside the cell.²

There are several reports on flavonoids acting as enzyme inhibitors.³ Ochiai et al.,⁴ demonstrated that green tea catechins can inhibit the activity of Na⁺, K⁺-ATPase in pig kidney. Catechins are known to protect the cardiovascular system. Their antihypertensive effect may be attributed to the inhibition of Na⁺, K⁺-ATPase.

Isoschaftoside (apigenin-6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranoside) has been described as a compound with allelopathic activity against Striga hermonthica, an obligate parasitic weed that can damage the maize crop⁵ and with nematocidal activity against the root-knot nematode (Meloidogyne incognita).⁶

The present study reports that isoschaftoside can inhibit Na⁺, K⁺-ATPase activity in pig kidney at different concentrations, although there are no data in the literature regarding effect of this compound on the pathology of hypertension.

Furthermore, this is the first study in which isoschaftoside was isolated from Syngonium podophyllum (Araceae), a medium-sized shrub distributed from Mexico to the Guianas, Bolivia, and Brazil. Leaves of S. podophyllum are used in traditional medicine in Central America to treat skin ailments, such as, wounds, dry skin, itching and rashes, whereas the leaf tincture is used to treat rheumatism, arthritis, pain, and swelling.⁷

MATERIAL AND METHODS

General experimental procedures

UV spectra were obtained using the DAD-UV detector (SP-M10A) of an HPLC system equipped with a Shimadzu LC-10AD. An RP 18 column (0.5mm x 250 x 4.6mm- Merck, Darmstadt, Germany) was used in the analyses. NMR spectra (¹H, 500 MHz; ¹³C, 125 MHz) were recorded in D₂O on a Bruker DRX spectrometer. HRMS/MS spectra were recorded on Finnigan TSQ Quantum Ultra AM, a triple quadrupole mass spectrometer operating in the electrospray ionization mode. HPLC-grade methanol (MeOH) and phosphoric acid were obtained from TediaBrazil. Deionized water (Milli- Q; Millipore) was used in the study. Thin-layer chromatography (TLC) was performed on silica gel GF²⁵⁴ Merck using butan-1-ol (BuOH); acetic acid (AcOH); H₂O (40:10:50, v/v) as the eluent, 1% “Natural Product Reagent” – (NP: 2-aminoethyl diphenylborinate – Sigma-Aldrich) in MeOH as a chromogenic reagent for flavonoids, and 5% polyethylene glycol-4000 (PEG) solution in EtOH as a visualization reagent under UV light (365 nm). Column chromatography was performed using cellulose-acetate (Sigma-Aldrich) and Sephadex LH-20 columns (Pharmacia). Ouabain was purchased from Sigma-Aldrich. Other reagents were purchased from Merck.

Plant material

S. podophyllum was collected in March 2009 at Vista Chinesa, Rio de Janeiro, Brazil, and identified by Prof. Dr. Cassia Sakuragui from the Biology Institute, Federal University of Rio de Janeiro. A voucher specimen (RB 480150) was deposited at the Herbarium of the Botanic Garden of Rio de Janeiro.

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**Extraction and isolation**

The air-dried leaves of *Syngonium podophyllum* (397 g) were extracted with EtOH:H₂O (7:3, v/v; 6 L × 5) at room temperature by static maceration for 15 days and concentrated to produce a dark green extract (45 g). The extract was suspended in MeOH:H₂O (9:1, v/v) and partitioned with hexane (200 mL × 3). The aqueous phase was concentrated to remove methanol, and successively partitioned with CHCl₃, ethyl-acetate and butan-1-ol (each 200 mL X 3). As this study aimed to isolate and elucidate the flavonoids in *S. podophyllum*, the BuOH extract (15 g) was chosen for the phytochemical investigation. This extract exhibited higher flavonoid content as indicated by the large spots on TLC plates after the treatment with the chromogenic reagent for flavonoids (NP/PEG). Hence, 7.5 g of the BuOH extract were chromatographed on a cellulose-acetate column (3.0 × 53.5 cm) with solvents elution under pressure, using a vacuum pump. The elution was performed in the gradient mode using H₂O and MeOH in different ratios (1.0, 8:2, 6:4, 4:6, 2:8 and 0:1, v/v). Fractions that eluted with water (Fr 4 to Fr 11) were combined because the presence of flavonoids in these fractions was demonstrated by TLC, and this combined fraction was subjected to column chromatography using a Sephadex LH-20 column (2.0 × 55 cm) as the stationary phase. Further elution was performed in the gradient mode using H₂O and MeOH in different ratios (1.0:9:1:8:2:7:3:6:4:5:5:4:6:3:7:2:8:1.9:0:1, v/v). Subfractions (Fr 10 to Fr 21) eluted with H₂O:MeOH (9:1, v/v) and H₂O:MeOH (8:2, v/v) were combined as Fr 1 because of their similarity on TLC. Some precipitation from Fr 1 was observed when methanol was used for solubilization. The precipitate was separated by centrifugation. TLC analysis suggested good purity of the precipitate and it was named as 1 (20 mg). After ¹H- and ¹³C-NMR analysis, using uni- and bidimensional techniques, I was identified as isoschaftoside as shown in Figure 1. Although the BuOH fraction displayed a high diversity of flavonoids, isoschaftoside was identified as the major component, and for the bioguided fractionation minor flavonoids were excluded. This study focused on the isolation and identification of the major flavonoid and the evaluation of its inhibitory activity on Na⁺, K⁺-ATPase.

**Basolateral membrane preparation**

Homogenized preparations of the basolateral membrane of the proximal tubule were obtained from adult pig kidneys. The kidneys were removed immediately after death of the animals and kept in an ice-cold solution containing: sucrose 250 mmol L⁻¹, HEPESTris (pH 7.6) 10 mmol L⁻¹, EDTA 2 mmol L⁻¹ and PMSF 1 mmol L⁻¹. Thin slices of the external cortex were removed and homogenized in the same solution using a Glass/Teflon homogenizer. The homogenate was centrifuged at 1.500 g at 4 °C, for 10 min. The precipitate was removed and the homogenate was collected and subjected to sequential centrifugations for basolateral membrane isolation. Initially, the homogenate was centrifuged at 13.000 g at 4 °C, for 44 min. After centrifugation, the supernatant was discarded and, using a solution containing sucrose 250 mmol L⁻¹ and EDTA, pH 7.6, the most superficial layer of the precipitated was collected, which generally corresponds to the microsomal fraction. Then, using a Percoll gradient (final concentration 12% v/v), the microsomal fraction was centrifuged at 40.000 g, at 4 °C, for 1h, to generate the Percoll density gradient, and consequently, to separate different membrane fractions contained in the brute portion of plasmatic membranes. Therefore, the fraction corresponding to basolateral membrane was collected, homogenized using a Glass/Teflon homogenizer and centrifuged at 200.000 g, for 1h, at 4 °C, for Percoll sedimentation. The membrane preparation was resuspended in sucrose 250 mmol L⁻¹ and the final total protein concentration was determined by the method given by Lowry et al. These samples were then stored in liquid nitrogen.

The Na⁺, K⁺-ATPase activity was determined by colorimetric detection of inorganic phosphate (Pi), resulting from ATP hydrolysis. The activity was determined by the calculating the difference between the absorbance values in the presence and absence of ouabain 2 mmol L⁻¹ (specific Na⁺, K⁺-ATPase inhibitor). The reaction medium contained Bis-Tris-propane 50 mmol L⁻¹ (pH 7.4), EDTA 0.2 mmol L⁻¹, MgCl₂ 5 mmol L⁻¹, NaCl 120 mmol L⁻¹ and protein. The membranes were pre-incubated in this medium along with flavonoids at 37 °C, for 10 min, to ensure a complete Na⁺, K⁺-ATPase modulation. The hydrolysis reaction was initiated by adding an ATP (5 mmol L⁻¹) and KCl (24 mmol L⁻¹) mixture, and it was terminated after 10 min by adding activated charcoal in HCl (0.1 mol L⁻¹).

**Statistical analysis**

Graphpad Prism® 5 software was used, and the results were subjected to analysis of variance (ANOVA). This study established a significance level of P < 0.05. The results are presented as the mean ± standard error of the mean.

**Isoschaftoside**

Isoschaftoside (apigenin-6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranosyde): amorphous yellow solid; ¹H- and ¹³C-NMR analysis, using uni- and bidimensional techniques, I was identified as isoschaftoside as shown in Figure 1. Although the BuOH fraction displayed a high diversity of flavonoids, isoschaftoside was identified as the major component, and for the bioguided fractionation minor flavonoids were excluded. This study focused on the isolation and identification of the major flavonoid and the evaluation of its inhibitory activity on Na⁺, K⁺-ATPase.

**RESULTS AND DISCUSSION**

**Isoschaftoside identification**

Isoschaftoside was obtained as an amorphous, yellow powder. The molecular formula was deduced from the HR-MS data as C₇₀H₅₇O₁₄ ([M-H] at m/z 563.14048). Its UV spectrum was same as flavone, with band I at 352 nm and band II at 299 nm. The ¹H- and ¹³C-NMR spectra were obtained in D₂O. These data are shown in Table 1, and they are compatible to those previously reported by Hooper et al.¹ C-glycosylation at the C-6 position was confirmed by HMBC correlations as shown in Figure 2. The anomeric hydrogen (H₁''') and H₁' correlation of 4.97 ppm established a ³JCa correlation with C-6 (108.64 ppm) and a ³JCa correlation with C-5 (160.29), suggesting arabinose as the carbohydrate located at C-6. Furthermore, a ³JCa correlation of 4.97 ppm with the carbon 67.91 ppm, a typical chemical shift of C-2 in arabinose, confirmed the identity of the sugar. Because of interference with the solvent signal, the correlation of H₁'''' with C-8 (103.95) could not be observed. Nevertheless, both isoschaftoside and schaftoside (glucose at C-6 and arabinose at C-8) feature a larger chemical shift on apigenin at C-6 than at C-8. Therefore, the flavone that was isolated from *S. podophyllum* concluded to be...
isoschaftoside. There have been previous reports concerning the occurrence of isoschaftoside in Philodendron sp. and Colocasia esculenta (Araceae),\textsuperscript{11,12} Desmodium uncinatum (Fabaceae),\textsuperscript{13} Mauritia flexuosa (Arecaceae),\textsuperscript{14} Saccharum officinarum and Triticum aestivum (Poaceae),\textsuperscript{15,16} Crataegus monogyna (Rosaceae)\textsuperscript{17} and Passiflora incarnata (Passifloraceae).\textsuperscript{18} Notably, antihypertensive activities of C. monogyna, C. esculenta and P. incarnata are known.\textsuperscript{19-21}

respectively. Even at a concentration of 0.25 mg mL\textsuperscript{-1}, isoschaftoside could decrease the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase activity by 39.75\% inhibition. At lower concentrations of 0.10 and 0.05 mg mL\textsuperscript{-1}, no inhibition was observed. Therefore, isoschaftoside inhibited renal Na\textsuperscript{+}, K\textsuperscript{+}-ATPase in a concentration-dependent manner.

Figure 3. Na\textsuperscript{+}, K\textsuperscript{+}-ATPase activity in presence of different Isoschaftoside concentrations (2.00 mg - 0.05 mg mL\textsuperscript{-1}). The graph shows 100\% activity in control. Results are expressed as mean ± S.D. Significance was determined by ANOVA followed by Tukey’s Multiple Comparison Test. Superscript letter a means significantly different from control. P < 0.05 vs. control

Table 1. \textsuperscript{1}H and \textsuperscript{13}C NMR assignment for Isochaftoside in D\textsubscript{2}O (\textsuperscript{1}H, 500 MHz; \textsuperscript{13}C, 125 MHz)

<table>
<thead>
<tr>
<th>Position</th>
<th>\textsuperscript{1}H (D\textsubscript{2}O - 25\textdegree C)</th>
<th>\textsuperscript{13}C (D\textsubscript{2}O - 25\textdegree C)</th>
<th>\textsuperscript{1}H (D\textsubscript{2}O - 25\textdegree C)</th>
<th>\textsuperscript{13}C (D\textsubscript{2}O - 25\textdegree C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>6.54 (s)</td>
<td>100.41</td>
<td>6.54</td>
<td>100.5</td>
</tr>
<tr>
<td>3</td>
<td>181.61</td>
<td>70.64</td>
<td>182.6</td>
<td>74.8</td>
</tr>
<tr>
<td>5</td>
<td>160.29</td>
<td>nd\textsuperscript{*}</td>
<td>157.68</td>
<td>108.64</td>
</tr>
<tr>
<td>6</td>
<td>108.64</td>
<td>nd\textsuperscript{*}</td>
<td>157.68</td>
<td>104.5</td>
</tr>
<tr>
<td>8</td>
<td>103.95</td>
<td>104.5</td>
<td>157.61</td>
<td>105.3</td>
</tr>
<tr>
<td>10</td>
<td>99.56</td>
<td>100.5</td>
<td>118.26</td>
<td>119.0</td>
</tr>
<tr>
<td>1'</td>
<td>118.26</td>
<td>119.0</td>
<td>129.01</td>
<td>129.1</td>
</tr>
<tr>
<td>2'</td>
<td>8.05 (d, 7.74Hz)</td>
<td>129.01</td>
<td>7.90 (d, 7.6Hz)</td>
<td>129.1</td>
</tr>
<tr>
<td>3'</td>
<td>6.83 (d, 7.83Hz)</td>
<td>118.19</td>
<td>6.82 (d, 8.3Hz)</td>
<td>118.9</td>
</tr>
<tr>
<td>4'</td>
<td>6.83 (d, 7.83Hz)</td>
<td>118.19</td>
<td>6.82 (d, 8.3Hz)</td>
<td>118.9</td>
</tr>
<tr>
<td>5'</td>
<td>8.05 (d, 7.74Hz)</td>
<td>129.01</td>
<td>7.90 (d, 7.6Hz)</td>
<td>129.1</td>
</tr>
</tbody>
</table>

\textsuperscript{6-C-\alpha-L-Ara}

1  | 4.97 (d, 9.47 Hz) | 75.09          | 4.88 (d, 9.4 Hz) | 75.3          |
2  | 4.63            | 67.91          | 4.76            | 67.9          |
3  | 3.71            | 74.85          | 3.78            | 74.7          |
4* | 4.05            | 68.47          | 4.09            | 69.8          |
5  | 3.95 and 3.72   | 70.64          | 4.03 and 3.85   | 70.4          |

\textsuperscript{8-C-\beta-D-Glu}

1'  | 5.08 (d, 9.43 Hz) | 75.63          | 5.22 (d, 9.9 Hz) | 74.8          |
2  | 4.03            | 69.79          | 4.33            | 71.0          |
3  | 3.62            | 74.60          | 3.6             | 79.1          |
4* | 3.95            | 69.64          | 3.81            | 69.9          |
5* | 3.72            | 78.98          | 3.7             | 81.5          |
6  | 3.68            | 61.08          | 4.01 and 3.88   | 61.9          |


**Assays for Na\textsuperscript{+}, K\textsuperscript{+}-ATPase**

As shown in Figure 3, isoschaftoside significantly decreased the activity of renal Na\textsuperscript{+}, K\textsuperscript{+}-ATPase at the concentrations of 2.00, 1.50, 1.00, 0.75 and 0.50 mg mL\textsuperscript{-1}, by 83.55\%, 72.85\%, 69.13\%, 66.70\% and 55.93\% (these values indicate the percentage change vs. control).
This is the first study to report the isolation of isoschaftoside from the genus *Syngonium*, and also suggest that this di-C-glycosylated flavone is a candidate compound for clinical assays involving primary hypertension because of its ability to inhibit renal Na\(^+\), K\(-\)ATPase, probably by a different mechanism than digitalic compounds. This disease is related to an excess of cellular sodium, vascular smooth-muscle cell contraction and peripheral vascular resistance. Further studies will be necessary to confirm this hypothesis.

**CONCLUSION**

The current study demonstrated that isoschaftoside considerably decreases renal Na\(^+\), K\(-\)ATPase activity. This compound is the major flavonoid produced by the plant and it can be used as a lead compound to further study the mechanisms of action of antihypertensive drugs other than digitalics.

**SUPPLEMENTARY MATERIAL**

\(^1\)H-NMR, DEPTQ and HRMS spectra for compound 1 are available in http://quimicanova.sbq.org.br, PDF format, with free access.

**ACKNOWLEDGMENT**

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**REFERENCES**

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Figure 1S. $^1$H NMR spectrum of isoschaftoside in D$_2$O at 25 °C (500MHz)
Figure 2S. Expansion of the aromatic hydrogens region. (D$_2$O, 500 MHz)

Figure 3S. Expansion of the anomeric hydrogens (D$_2$O, 500 MHz)
In vitro effect of isoschaftoside isolated from *Syngonium podophyllum* on pig kidney Na\(^+\), K\(^+\)-ATPase

**Figure 4S.** DEPTQ spectrum of isoschaftoside in D\(_2\)O at 25 ºC (125MHz)

**Figure 5S.** HR-ESIMS (negative mode) of isoschaftoside