The effect of saponins from *Ampelozizyphus amazonicus* Ducke on the renal Na\(^+\) pumps' activities and urinary excretion of natriuretic peptides

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**Abstract**

**Background:** In a previous study, we showed that a saponin mixture isolated from the roots of *Ampelozizyphus amazonicus* Ducke (SAP\(^{AaD}\)) reduces urine excretion in rats that were given an oral loading of 0.9 % NaCl (4 ml/100 g body weight). In the present study, we investigated whether atrial natriuretic peptides (ANP) and renal ATPases play a role in the SAP\(^{AaD}\)-induced antidiuresis in rats.

**Methods:** To evaluate the effect of SAP\(^{AaD}\) on furosemide-induced diuresis, Wistar rats (250-300 g) were given an oral loading of physiological solution (0.9 % NaCl, 4 ml/100 g body weight) to impose a uniform water and salt state. The solution containing furosemide (Furo, 13 mg/kg) was given 30 min after rats were orally treated with 50 mg/kg SAP\(^{AaD}\) (SAPA\(^{aD} + \)Furo) or 0.5 ml of 0.9 % NaCl (NaCl + Furo). In the SAP\(^{AaD} + \)NaCl group, rats were pretreated with SAP\(^{aD}\) and 30 min later they received the oral loading of physiological solution. Animals were individually housed in metabolic cages, and urine volume was measured every 30 min throughout the experiment (3 h). To investigate the role of ANP and renal Na\(^+\) pumps on antidiuretic effects promoted by SAP\(^{aD}\), rats were given the physiological solution (as above) containing SAP\(^{aD}\) (50 mg/kg). After 90 min, samples of urine and blood from the last 30 min were collected. Kidneys and atria were also removed after previous anesthesia. ANP was measured by radioimmunoassay (RIA) and renal cortical activities of Na\(^+\)-ATPase (from 25.0 ± 5.9 nmol Pi.mg\(^{-1}\).min\(^{-1}\), control, to 52.7 ± 8.9 nmol Pi.mg\(^{-1}\).min\(^{-1}\), p < 0.05) and (Na\(^+\),K\(^+\))-ATPase (from 47.8 ± 13.3 nmol Pi.mg\(^{-1}\).min\(^{-1}\), control, to 79.8 ± 6.9 nmol Pi.mg\(^{-1}\).min\(^{-1}\), p < 0.05) activities in the renal cortex. SAP\(^{aD}\) also lowered urine ANP (from 792 ± 132 pg/mL, control, to 299 ± 88 pg/mL, p < 0.01) and had no effect on plasma or atrial ANP.

**Conclusion:** We concluded that the SAP\(^{aD}\) antidiuretic effect may be due to an increase in the renal activities of Na\(^+\)- and (Na\(^+\),K\(^+\))-ATPases and/or a decrease in the renal ANP.

**Keywords:** *Ampelozizyphus amazonicus* Ducke, Rhamnaceae, saponins, antidiuresis, Na\(^+\)-ATPase, (Na\(^+\),K\(^+\))-ATPase, atrial natriuretic peptides

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Background
Several studies have reported that some medicinal herbs alter diuresis [1-5] even though little is known about the mechanism(s) that account for this claimed physiological effect. *Ampelozizyphus amazonicus* (*A. amazonicus*) is a Rhamnaceae known as ‘saracura-mirá’ or ‘Indian beer’ that is traditionally used by Brazilian Indians and Cabocos (from the Tupi *kaa'boc* who live in all regions along Rio Negro, Amazônia. Beverages prepared with their roots are used as stimulant against tiredness, fatigue and starvation [6]). The beverage is also used against malaria, liver disturbances and sleeplessness, and serve as depurative [7].

In a previous studies, we have shown that a triterpene saponin mixture isolated from the roots of *A. amazonicus* Ducke (SAPaD), reduced the urine excretion of normal rats in a dose-dependent manner [8]. There is a need for novel oral antidiuretics to treat diseases, such as pituitary diabetes insipidus and nephrogenic diabetes insipidus; however, investigations for compounds other than the typically used arginine vasopressin (AVP) or desmopressin are lacking. It is well known that diuresis is regulated by a number of endogenous and exogenous compounds that act at multiple intrarenal sites to influence urine formation [9]. The final urine volume and composition are determined primarily in the renal tubule system without a direct extensive dependence on the glomerular filtration rate. In the renal tubules, water reabsorption is usually secondary to transcellular sodium reabsorption. Additionally, AVP regulates water balance and osmolality by manipulating water permeability that is not obscured by responses to sodium reabsorption [10]. Renal tubular sodium reabsorption involves two primary active transporters: the ouabain-sensitive (Na⁺,K⁺)-ATPase and the furosemide-sensitive Na⁺-ATPase [9-11]. The activity of both ATPases is directly regulated by hormones, such as natriuretic peptides. These hormones play an important role in hydro-electrolytic homeostasis by stimulating natriuresis through the coordination of apical sodium channels and basolateral (Na⁺,K⁺)-ATPases in the inner medullary collecting ducts [12-16]. Over the last few decades, several studies have demonstrated the potential role of the cortical sodium pumps in the composition of excreted urine. The various hormones that play a role in the modulation of urine composition act on these sodium pumps in the cortical segment, particularly in the proximal tubule [17-19]. Accordingly, it is well known that specific modifications of sodium reabsorption in the proximal tubule cells lead to an increase in renal sodium and water excretion as observed in primary hypertension [20-23]. Then, it is plausible to postulate that changes in the cortical sodium pumps promoted by a compound could lead to changes in urine composition. It has been reported that sodium pumps’ activities can be modulated by herbal products, such as *Petroselinum hortense* extracts, which inhibit (Na⁺, K⁺)-ATPase, and saponins isolated from *Costus spicatus* Swartz, which inhibit Na⁺-ATPase activity [24,25]. Because the inhibition of renal Na⁺ transport may account for the diuretic effect of different agents, we investigated the renal cortical Na⁺ pumps and renal natriuretic peptides in SAPaD-induced antidiuresis.

Methods
Plant material
The roots of *A. amazonicus* Ducke were collected (September, 2000) in the city of Presidente Figueiredo, Amazonas State, Brazil, and were identified by Dr. Ari Hidalgo. Voucher specimens (189,858) were deposited in the herbarium of the Instituto Nacional de Pesquisa da Amazônia (Manaus/AM, Brazil).

Extraction, isolation and characterization of SAPaD
The extraction, isolation and chemical characterization of SAPaD were performed according to previously described procedures [8]. Powdered roots of *A. amazonicus* were successively extracted by percolation with 70 % ethanol, with the solvent then evaporated to dryness (6.6 % w/w). The crude extract was resuspended in water and treated with n-butanol. After evaporation at a maximum of 60°C, the organic and aqueous phases furnished the SAPaD and saponin-free (SAPaD-free) fractions, respectively. Chemical characterisation was performed by HPLC/DAD. Briefly, HPLC analysis was carried out on an Agilent 1200 system (Palo Alto, CA, USA). Column, lichrospher reversed-phase C18 (250 mm x 4.6 mm I.D., 5 µm particle size, Merck); mobile phase, acetonitrile (solvent A) and water (solvent B); elution gradient, 30–40 % A at 0–10 min, 40–90 % A at 10–40 min, 90–100 % A at 40–45 min, and 100 % A at 45–50 min; flow rate, 1.0 mL/min; injection volume, 20 µL. For the analysis, 30 mg of SAPaD were dissolved in 10 mL of acetonitrilewater (30:70) in an ultrasonic bath for 10 min. All solutions were filtered through a 0.45 µm membrane filter before injection. UV photodiode array detection was performed at 205 nm, and UV spectra from 200 to 400 nm were on-line recorded for peak identification.

Animals
Male Wistar rats weighing 250–300 g were housed in standard conditions with free access to commercial chow and water prior to the experiment. Animals were kept at a room temperature of 22°C with a light/dark cycle of 14/10 h. All procedures described here had prior approval from the Institutional Animal Use Ethics Committee (protocol 177, November, 2008).

Interaction between furosemide and SAPaD
To evaluate a possible interaction between furosemide (Lasix®, Aventis Pharma Ltda, São Paulo, Brazil) and
SAPAd, all animals were given an oral loading of physiological solution (0.9 % NaCl, 4 ml/100 g body weight) to impose a uniform water and salt state. The physiological solution containing furosemide (13 mg/kg) was given 30 min after rats were orally treated with 50 mg/kg SAPAd (SAPAd + Furo group) or 0.5 ml of 0.9 % NaCl (NaCl + Furo group). Rats of group SAPAd + NaCl were pretreated with SAPAd and 30 min later they received the oral loading of physiological solution. Rats in the NaCl + AVP group received the physiological solution containing 200 ng/kg AVP (Sigma Chemical Co., St Louis, MO, USA, 98 % purity) after (30 min) rats were orally treated with 0.5 ml of 0.9 % NaCl. Rats in the NaCl + NaCl group received the oral loading of physiological solution after pretreatment with 0.5 ml of 0.9 % NaCl (control). Animals were individually housed in metabolic cages, and urine volume was measured every 30 min throughout the experiment (3 h).

In a series of experiments, rats were given the oral loading of physiological solution as above in the absence (control) and presence of 50 mg/kg SAPAd. At 90 min after SAPAd administration, the urine volume was measured, and a sample of urine was collected and stored at −20°C until the measurement of urine ANP was performed. Afterward, the rats were anesthetized with 40 mg/kg of thiopental, and a blood sample was drawn from the inferior cava vein. Under anesthesia, the rats were sacrificed, and the kidneys were removed for the histomorphological analysis and the renal ATPase activity measurements. Both atria were also removed to determine the ANP content. Blood samples were centrifuged at 3,000 rpm for 10 min at 4°C, and the recovered plasma was stored at −80°C until the ANP assay was performed. For the histomorphological analysis, sections of the kidney were obtained from representative animals in the control and SAPAd-treated groups. The tissue was fixed in 10 % formalin, embedded in paraffin and was dissected into 4 μm sections. The sections were stained with hematoxylin and eosin and were examined under a light microscope.

Measurement of atrial natriuretic peptides
To determine the atrial content of ANP, the left and right atria were pooled in a prechilled tube containing a cocktail of protease inhibitors consisting of 0.1 M acetic acid, 10−5 mol/L EDTA, 10−5 mol/L PMSF and 0.5 x 10−5 mol/L pepstatin A (Sigma Chemical Co., St Louis, MO, USA). The tube contents were homogenized (Euroturrax homogenizer T20b; Janke and Kunkel Ika Labortechnik, Staufen, Germany) and centrifuged at 20,000 x g for 30 min at 4°C. The supernatants were diluted in a phosphosaline buffer (0.01 mol/L sodium phosphate, 0.14 mmol/L bovine serum albumin, 0.10 % Triton X-100, 0.10 mol/L NaCl and 0.01 % sodium azide, pH 7.4), and ANP content was determined by radioimmunoassay (RIA) [29]. ANP antibody was kindly donated by Dr. J Gutkowska (CHUM – Université de Montreal, Montreal, Canada). Standard ANP was obtained from Bachem Inc. (Torrance, CA, USA). The protein concentration in the supernatant was measured as previously described [30]. Atrial ANP was normalized to protein concentration and reported in micrograms per milligram of protein. To quantify plasma ANP, plasma samples (1 mL) were extracted in Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA). The protein concentration in the supernatant was determined using anti-ANP antibodies. Because atrial ANP and urine ANP have a very similar structure with an identical C-terminus [31], anti-ANP antibodies may also be employed to measure ANP in urine. In addition, insignificant concentrations of circulating ANP are found in the urine because of high ANP degradation from peptidases in the kidney cortex membranes or because of ANP binding to the clearance C receptor [32,33].

Statistical analysis
The results were expressed as means ± standard error of the mean (± SEM). The data shown the effect of
SAPaD and furosemide on diuresis were analyzed using nonlinear regression and two-way ANOVA followed by Bonferroni’s test. To analyze the other investigated parameters, unpaired Student’s t-test was also employed to compare the control and SAPaD-treated groups. A p-value less than 0.05 (p < 0.05) was considered statistically significant.

Results
Figure 1 shows a typical fingerprint chromatogram of SAPaD mixture. The retention times of saponins were between 9.42 and 16.78 min. The purification process performed to obtain saponins was very efficient since the peaks related to saponins were predominant in the SAPaD fraction fingerprint.

All experiments using animals were performed in rats with a 4 % body weight volume expansion (oral loading of 0.9 % NaCl, 4 ml/100 g body weight) because this magnitude of volume expansion is an intermediate value between the experimentally obtainable minimum and maximum values used for studies examining the acute effect of substances that either stimulate or inhibit diuresis. In this study, SAPaD was tested at the dose of 50 mg/kg because it resulted in an approximate 60 % reduction in the diuresis induced by the oral loading of 0.9 % NaCl (4 ml/100 g body weight) in normal rats. This reduction was reported in our previous study [8] in which we have shown that SAPaD at dose varying from 25 to 1000 mg/kg reduced the urine production in a dose-dependent manner. As 60 % reduction neither represent the minimum nor the maximum effect of SAPaD, 50 mg/kg was the dose of choice to perform the experiments in the present study. No significant alteration of renal morphology was observed after SAPaD treatment (data not shown).

Interaction between furosemide and SAPaD
As shown in Figure 2, SAPaD and furosemide have antagonistic effects on diuresis. While SAPaD reduced the diuresis elicited by the oral loading of 0.9 % NaCl (at 180 min: from 8.5 ± 1.0 mL, NaCl + NaCl group, to 4.0 ± 1.3 mL, SAPaD + NaCl group, n = 5) (Figure 2A), furosemide dosed at 13 mg/kg greatly increased urine elimination in rats that equally received oral 0.9 % NaCl (at 180 min: from 8.5 ± 1.0 mL to 12.2 ± 1.4 mL, NaCl + Furo group, n = 5) (Figure 2A). However, pretreatment with SAPaD eliminates this increase in the furosemide-induced diuresis (Figure 2B). In the presence of SAPaD, the urine volume produced by furosemide (8.5 ± 1.3 mL, SAPaD + Furo group n = 5) (Figure 2B) was similar to that observed in the NaCl + NaCl group (8.5 ± 1.0 mL, n = 5) (Figure 2A) at 180 min. It is noteworthy that SAPaD, although in a less extent, induced antidiuresis as AVP, which was used as a positive control (Figure 2B).

Effect of SAPaD on renal ATPases’ activities and atrial natriuretic peptides
SAPaD (50 mg/kg) significantly increased the activities of renal Na+-ATPase (from 25.0 ± 5.9 nmol Pi. mg\(^{-1}\). min\(^{-1}\), control, n = 4, to 52.7 ± 8.9 nmol Pi.mg\(^{-1}\).min\(^{-1}\), n = 4, p < 0.05) (Figure 3A) and renal (Na\(^{+}\), K\(^{-}\))-ATPase (from 47.8 ± 13.3 nmol Pi.mg\(^{-1}\).min\(^{-1}\), control, n = 4, to 79.8 ± 6.9 nmol Pi.mg\(^{-1}\).min\(^{-1}\), n = 4, p < 0.05) (Figure 3B). The ANP levels in both the plasma (Figure 4A, n = 6) and atria (Figure 4B, n = 6) were not significantly affected by SAPaD at the 50 mg/kg dosage. In contrast, urine ANP excretion was significantly reduced in the rats treated with SAPaD (from 792 ± 132 pg/mL, control, n = 7, to 299 ± 88 pg/mL, n = 7, p < 0.01) (Figure 4C).

Discussion
In this study, we showed for the first time that saponins, in vivo, stimulate renal ATPases and reduce the level of urine ANP. These effects are undoubtedly characteristic of an antidiuretic agent. Moreover, SAPaD abolished the diuretic effect of furosemide, a classical diuretic that affects renal Na+-ATPases [32]. In a previous study, our group reported that the oral administration of SAPaD exhibited a dose-dependent antidiuretic effect in normal rats in either dehydrated or hydrated conditions [8]. In the present study, SAPaD exhibited a similar effect even when administered 30 min prior to an oral loading of physiological solution (0.9 % NaCl). Because pretreatment with SAPaD prevented the effect of furosemide, one could expect that SAPaD might share the same mechanistic pathway by which furosemide enhances diuresis. According to Becker et al. [34], the diuretic effect of furosemide was reduced in hypertensive patients with domiciliary use of a nutritional supplement derived from ginseng. Ginseng is a natural compound that is rich in triterpene saponins structurally similar to SAPaD. It is known that furosemide inhibits renal Na\(^{+}\)-ATPase

![Figure 1 HPLC fingerprinting of SAPaD. The retention times of saponins are (in min): 1–9.42, 2–9.85, 3–11.32, 4–13.46, 5–13.79, 6–14.75 and 7–16.78. Detection, 205 nm; mAU, milli absorbance unit.](http://www.biomedcentral.com/1472-6882/12/40)
activity [11,32]. In addition, some studies have reported that the activities of renal ATPases may be modulated by medicinal plants. It has been shown that the diuretic effect of crude *Petroselinum hortense* extract is accompanied by the inhibition of (Na⁺,K⁺)-ATPase activity [25]. Na⁺-ATPase was similarly inhibited by steroidal saponins isolated from *Costus spicatus*, a plant used in Brazilian folk medicine to expel kidney stones [24]. In contrast, the present study shows SAPAaD in vivo produced a significant increase in both Na⁺- and (Na⁺,K⁺)-ATPase activities. These data suggest that SAPAaD decreased the diuresis, at least in part, by stimulating renal ATPases. Accordingly, it was previously reported that saponins un-mask a latent intracellular pool of (Na⁺,K⁺)-ATPase leading to an increase in the renal tubular ATPase activity [35,36]. It has been reported that endogenous compounds, such as ANP99-126 and urodilatin [12-14], stimulate natriuresis and diuresis primarily by coordinating the inhibition of apical Na⁺ channels and basolateral (Na⁺,K⁺)-ATPases in the inner medullary collecting ducts [12]. However, a possible role of the cortical sodium pumps in the urine final composition cannot be ruled out. Different hormones that modulate the urine composition act on these cortical sodium pumps located particularly in the proximal tubules [17-19]. It is well established that specific modifications in sodium re-absorption in proximal tubule cells lead to an increase in renal sodium and water excretion as observed in primary hypertension [20-23]. These observations indicate that changes in the cortical sodium pumps may lead to changes in urine composition. In addition, alteration in the renal and/or plasma levels of the natriuretic peptides may play a role in the SAPAaD-induced antidiuresis. A significant reduction in the urine ANP concentration was observed in the urine of rats treated with SAPAaD. Therefore, we cannot exclude the possibility that the increase in renal ATPase activity induced by SAPAaD was a result of the reduction in the renal urodilatin level.

![Figure 2](image1)

**Figure 2** Effect of SAPAaD and furosemide on diuresis in rats. Animals were given an oral loading of physiological solution (0.9 % NaCl, 4 ml/100 g body weight) to impose a uniform water and salt state. In **A**: Rats were orally pretreated (30 min) with 0.5 ml 0.9 % NaCl and received the physiological solution alone (NaCl + NaCl) or containing 13 mg/kg furosemide (NaCl+ Furo); SAPAaD + NaCl, rats were orally pretreated with 50 mg/kg SAPAaD and received the physiological solution alone. In **B**: Rats were orally pretreated with SAPAaD and received the physiological solution containing furosemide (SAPAaD + Furo); NaCl + AVP, rats were orally pretreated with 0.5 ml 0.9 % NaCl and received the physiological solution containing AVP. NaCl+Furo is the same as in **A**. Animals (n = 5/group) were individually housed in metabolic cages, and urine volume was measured every 30 min throughout the experiment (3 h). Data are expressed as means ± SEM. In **A**, *p < 0.05 vs. group NaCl + NaCl. In **B**: *p <0.05, **p < 0.01 and ***p < 0.001 vs. group NaCl + Furo at the correspondent time. Data were analyzed by two-way ANOVA followed by Bonferroni’s test.

![Figure 3](image2)

**Figure 3** Effect of SAPAaD on renal Na⁺- (A) and (Na⁺, K⁺)-ATPase activities (B). Rats were given an oral loading of physiological solution (0.9 % NaCl, 4 ml/100 g body weight) in the absence (control) and presence of 50 mg/kg of SAPAaD. At 90 min after SAPAaD administration, rats were sacrificed, and the kidneys were removed for renal cortical ATPase measurement. ATPases’ activities were expressed as nmol Pi per milligram of total protein per minute. Data are expressed as means ± SEM of four rats/group. *p < 0.05 compared with the control group, unpaired Student’s t-test.
modulate the activity of renal cortical Na\(^+\) pumps result-
tent. In turn, the low level of renal ANP would not
influence the urinary elimination of salts and water. In our study, the reduction in the
K\(^+\)\)-ATPase activity was measured in the whole cortex
and cortex of Sprague–Dawley rats. The discrepancy between
this study and our investigation may be because (Na\(^+\),
K\(^+\))\)-ATPase activity was measured in the whole cortex
and because the urine ANP excretion was lower than the
exogenous urodilatins used in the previous study (10
mM). Natriuretic peptides are very important hormones
that regulate renal processes since they are effective at
promoting changes in renal vasculature by acting as
vasodilators, and renal tubules by reducing Na\(^+\) and
water reabsorption. In our study, the reduction in the
urine ANP may denote a decreased intrarenal ANP con-
tent. In turn, the low level of renal ANP would not
modulate the activity of renal cortical Na\(^+\) pumps resulting
in a higher Na\(^+\) reabsorption. As a consequence, the
urinary elimination of salts and water is diminished.

Conclusions
Although the precise mechanism by which SAPAaD
affect diuresis is not completely elucidated, our data indi-
cate that the antidiuretic effect of SAPAaD may be due to
a reduction in the renal ANP levels and/or an increase in
the renal ATPase activity. We also conclude that sapo-
nins from A. amazonicus Ducke might be an herbal can-
didate for an antidiuretic with therapeutic potential. On
the other hand, the A. amazonicus Ducke species should
be used with caution because antidiuretic agents should
be avoided in certain conditions, such as hypertension.

Abbreviations
ANP: atrial natriuretic peptide; AVP: arginine vasopressin; Furo: furosemide;
HPLC/DAD: high performance liquid chromatography with diode array
detectors; SAPAaD: saponin mixture isolated from the roots of
Ampelozizyphus amazonicus Ducke.

Figure 4 Effects of SAPAaD on plasma (A), atria (B) and urine (C) ANP. Rats received an oral loading of physiological solution (0.9 % NaCl,
4 ml/100 g body weight) in the absence (control) and presence of 50 mg/kg of SAPAaD). At 90 min after SAPAaD administration, a sample of
blood and urine were collected, rats were sacrificed and the atria were removed for ANP measurement. Plasma (n = 6), pooled left and right atria
(n = 6) and urine ANP (n = 7) were measured by RIA. Atria ANP were normalized to protein concentration and reported in micrograms per
milligram of protein. Data are represented as means ± SEM. ANP in control: Plasma and atria (n = 12) and urine (n = 7). **p < 0.01 compared with
the control group, unpaired Student’s t-test.

Competing interests
The authors declare that they have no competing interests.

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Author’s contributions
LRLD designed and performed all of the experimental protocols, took part in
all of the analytical procedures, analysed the data and prepared the
manuscript; VGP designed and performed the RIAs analysis and analysed the data; MdGLB
designed the analytical protocol to measure the renal Na+-pumps activities; CCN
and prepared the manuscript; GDC, performed the histomorphological
analysis; AMdR designed the RIAs analysis and analysed the data; MdGLB
designed and supervised the experiments, analysed the data and prepared
the manuscript. All authors read and approved the final manuscript.

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