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Preclinical evaluation of the diuretic and saluretic effects of (-)-epicatechin and the result of its combination with standard diuretics

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ABSTRACT

Several studies have suggested that (-)-epicatechin-containing foods and plant extracts benefit conditions that affect the cardiovascular system, such as hypertension and endothelial dysfunction. However, no study was conducted so far to evaluate the potential of this flavonoid on diuretic activity assay. For that, female Wistar normotensive (NTR) and spontaneously hypertensive rats (SHR) received a single oral treatment with (-)-epicatechin (EPI), hydrochlorothiazide (HCTZ) or just vehicle (VEH). The effects of EPI in combination with diuretics for clinical use, as well as with L-NAME, atropine and indomethacin were also explored. Cumulative urine volume, plasma and urinary parameters were evaluated at the end of 8 h experiment. When given to NTR and SHR, at doses of 0.3, 1 and 3 mg/kg, EPI was able to stimulate both diuresis and saluresis (Na^+, K^+ and Cl^-), without interfering with plasma electrolyte content or urinary pH and uric acid values, when compared with VEH-treated only rats. The combination with HCTZ, but not with furosemide or amiloride, successfully strengthened EPI-induced diuresis. This effect was not accompanied by a potentiation of the saluretic effects. On the other hand, when given EPI in combination with amiloride, a significant increase in Cl^- excretion and maintenance of the potassium-sparing effects characteristic of this class of diuretics were detected. In addition, the diuretic effect of EPI was enhanced after pretreatment with L-NAME and its action was significantly precluded in the presence of indomethacin, a cyclooxygenase inhibitor. In conclusion, this study shows the diuretic and saluretic properties of EPI in rats, adding another biological activity whose effect may contribute to the different positive actions already described.

1. Introduction

(-)-Epicatechin, a plant based phytochemical classed as a flavonoid (in particular the subtype known as flavanols), is one of several types of bioactive constituents found in *Camellia sinensis*, *Vitis vinifera* and *Theobroma cacao*, popularly known as green tea, grape and cocoa, respectively [1–3]. A number of controlled human studies with flavanol-rich preparations point toward a negative correlation between the consumption of flavonols and the incidence of cardiovascular events, following features of modifying lipid metabolism, regulating insulin sensitivity, vascular endothelial protection, and reducing blood pressure [4–8].

In addition to the studies described above, the actions of the isolated (-)-epicatechin on experimental animal models have also been explored. For instance, the cardioprotective effect of (-)-epicatechin was demonstrated in animal models of ischemia-reperfusion [9–11]; the effects on the improvement in endothelial function was described in diabetes model [12], in the deoxycorticosterone acetate-salt-induced hypertension [13] and in the prothrombotic state associated with obesity-induced hypertension [14]. Moreover, there are several descriptions about its antioxidant effects [15], as well as renal protective effect in streptozotocin-induced diabetes model [16], in cisplatin-induced nephropathy [17], and against lipopolysaccharide-induced renal inflammation [18].

Nowadays, several vegetal drugs containing flavonoids are studied due to its ability to increase the volume of urine and electrolyte excretion, helping the body to reduce fluid buildup. The most common condition treated with diuretics agents is high blood pressure. Furthermore, diuretics drugs are also useful for the treatment of edema-related disorders as the congestive heart failure. In this context, the

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search for new therapeutic alternatives for cardiovascular and renal disorders is extremely necessary, both to broaden the available pharmacological options and to prove the effectiveness of preparations used in folk medicine. Therefore, although previous studies investigating flavanol-rich plants and foods provide indications for potential cardio and renal protective effects, the effects of individual flavanols remain unclear. Therefore, this study was proposed to investigate the diuretic and saluretic properties of (-)-epicatechin in both normotensive and hypertensive rats, as well as elucidates its main mode of action.

2. Material and methods

2.1. Isolation and identification of (-)-epicatechin

The branches of *Garcinia achachairu* were submitted to the maceration process to obtain the crude extract, followed by liquid:liquid partitions to obtain the fractions. A detailed description of the process of obtaining the extracts and fractionation is available in Mariano et al., [19]. Briefly, the ethyl acetate fraction (25.72 g) was subjected to column chromatography (0.063–0.20 mm, 242.0 g, 5.5 x 50 cm, Merck) over silica-gel and eluted with chloroform:methanol (100:0 → 0:100) in increasing order of polarity to afford 87 fractions, which were combined based on TLC profiles. The fraction 15–30 (3.74 g) was re-chromatographed using a solvent system of chloroform: methanol, yielding new 82 sub-fractions. Sub-fraction 70–78 eluted with a mixture of chloroform: methanol (80:20), presented as an off-white powder (229.81 mg) and was identified as (-)-epicatechin (EPI) by nuclear magnetic resonance (NMR) and spectrum using polarization transfer (DEPT) data. The data were compared with previous records described by Lopes et al., [20].

2.2. Conditions of HPLC analysis

The equipment Shimadzu® LC 20-AT system was used for HPLC analysis, consisting of a quaternary pump and a Shimadzu SPD-M20 A photodiode array detector, SIL-20 A HT auto-sampler and for the chromatogram registration a software LC Solution. The chromatographic column used was Phenomenex C18 column (250 × 4.6 mm) with core-shell particles of 5.0 µm and temperature at 35 °C. The method was chosen according to the best resolution of chromatogram of fraction ethyl acetate and (-)-epicatechin at a concentration of 2 and 0.5 mg/ml in methanol solution, respectively. All solutions were filtered through 0.45 µm membrane. The best mobile phase was acetonitrile and water at a flow rate of 1 mL/min, which the gradient elution was programmed as follows: initial isocratic condition during 15 min (10:90), 15–30 min (30:70) and 30–40 min (10:90). All the analysis were monitored at 205 nm.

2.3. Animals

This study used female Wistar normotensive and spontaneously hypertensive rats (SHR), 3–4 months old, delivered by Universidade do Vale do Itajaí (UNIVALI). The animals were maintained under standard conditions, 12 h light/dark cycle, temperature of 22 ± 2 °C, with free access to water and food. All the procedures were approved by institutional ethics committee from UNIVALI (license no. 028/17p) and were executed in accordance with all ethical standards and animal care established.

2.4. Diuretic activity assay

One day before the experiment, all animals were weighed and kept for 2 h in the metabolic cages for acclimation, followed by food fasting at 12 h with water ad libitum (and throughout the experiment). The rats (n = 6–8) were randomly distributed into groups and received 5 mL/100 g of physiological saline solution to obtain sodium and water body uniformity. In sequence, they were orally treated by gavage with vehicle (water plus 0.5% tween 80; 10 ml/kg), hydrochlorothiazide (HCTZ; 10 mg/kg) or (-)-epicatechin (EPI; 0.3, 1 or 3 mg/kg). Immediately after treatment, the animals were individually placed in metabolic cages where they were kept for 8 h, having urine collected in the first and every 2 h. Cumulative urine volume was expressed as mL/100 g. The values of pH, osmolality, electrolyte excretion (Na^+, K^+ and Cl^-) and uric acid were measured in each urine sample. Blood samples were also obtained at the end of the experiment and used to determine plasma electrolyte values. A detailed description of all the analytical protocols used in this study can be seen in de Souza et al. [21].

The effects of EPI in combination with other diuretics, as well as with drugs that interfere with the action of endogenous vasodilator mediators, were also evaluated. For that, HCTZ (10 mg/kg), furosemide (10 mg/kg), amiloride (3 mg/kg), L-NAME (60 mg/kg), atropine (1.5 mg/kg), indomethacin (5 mg/kg), or just vehicle (10 ml/kg; water plus 0.5% tween 80) was given to rats prior the treatment with EPI (3 mg/kg) or vehicle. The subsequent protocols adopted here followed as explained above. All drugs and doses used herein were chosen from previous studies [22–25].

2.5. Statistical analysis

The program GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA) was used to assemble the figures and perform the statistical analysis, by using one-or two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The data were expressed as mean ± standard error of mean (S.E.M) of 6–8 animals per group and a value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

The compound object of the present study was isolated from the ethyl acetate fraction (AcEt) from *G. achachairu* branches, and identified as (-)-epicatechin accordingly with NMR and DEPT data in comparison with previously reported by Lopes et al., [20], as follow: ^{1}H NMR (300 MHz, CD_3OD): 4.81 (s, H-2,), 4.17 (m, H-3), 2.70 (dd, H-4a, *J* 3.0, 2.7), 2.84 (dd, H-4b, *J* 4.8, 4.2), 5.93 (d, H-6, *J* 2.1), 5.91 (d, H-8, *J* 2.1), 6.97 (d, H-2′, *J* 1.5), 6.78 (d, H-5′), 6.81 (d, H-6′, *J* 1.8). ^{13}C NMR (75 MHz, CD_3OD): 80.0 (C-2), 67.6 (C-3), 29.4 (C-4), 158.1 (C-5), 96.5 (C-6), 157.8 (C-7), 96.0 (C-8), 157.5 (C-9), 100.2 (C-10), 132.4 (C-1′), 115.4 (C-2′), 146.9 (C-3′), 145.9 (C-4′), 116.0 (C-5′), 119.5 (C-6′) [20]. EIMS *m/z* 289 [M] +; (calcd for $C_{15}H_{13}O_6$). In addition, Fig. 1 shows the comparative HPLC analysis between AcEt fraction and the isolated (-)-epicatechin (EPI), which proves to be the major constituent found in this fraction. The compound, which is represented in the upper part of Fig. 1, showed a high degree of purity (> 97%). It is important to point out that EPI, and other compounds of the catechin family, have attracted great interest because they can be found in high concentrations in certain fruits, vegetables [26] and plant extracts (as described herein). In addition, they are readily absorbable by being found in aglycan form; i.e. not bound to sugars [27], which also emphasizes its importance when applied to in vivo biological tests.

Starting the biological assays, we initially accessed the effects of a single-dose treatment with different doses of EPI on the urinary volume of normotensive and hypertensive rats, as displayed in Fig. 2. The SHR is an inbred strain established from outbred normotensive ancestors Wistar rats selected for high blood pressure. It is the most commonly used animal model of hypertension and is frequently used to study pressure-dependent kidney damage [28,29]. When orally given to normotensive and hypertensive rats, at doses of 0.3, 1 and 3 mg/kg, EPI was able to intensify urine excretion, showing significant results already at the beginning of the 1st hour and remaining until the end of the 8th hour. The results were similar to those obtained from the group receiving hydrochlorothiazide (HCTZ), a thiazide-type reference