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Inhibition of angiotensin convertin enzyme (ACE) activity by the anthocyanins delphinidin- and cyanidin-3-*O*-sambubiosides from *Hibiscus sabdariffa*

Deyanira Ojeda[a], Enrique Jiménez-Ferrer[b], Alejandro Zamilpa[b], Armando Herrera-Arellano[b], Jaime Tortoriello[b], Laura Alvarez[a,*]

[a] *Centro de Investigaciones Químicas, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001, Chamilpa, Cuernavaca, 62209 Morelos, Mexico*
[b] *Centro de Investigación Biomédica del Sur, Instituto Mexicano del Seguro Social (IMSS), Xochitepec, Morelos, Mexico*

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ABSTRACT

Ethnopharmacological relevance: The beverages of *Hibiscus sabdariffa* calyces are widely used in Mexico as diuretic, for treating gastrointestinal disorders, liver diseases, fever, hypercholesterolemia and hypertension. Different works have demonstrated that *Hibiscus sabdariffa* extracts reduce blood pressure in humans, and recently, we demonstrated that this effect is due to angiotensin converting enzyme (ACE) inhibitor activity.
Aim of the study: The aim of the current study was to isolate and characterizer the constituents responsible of the ACE activity of the aqueous extract of *Hibiscus sabdariffa*.
Materials and methods: Bioassay-guided fractionation of the aqueous extract of dried calyces of *Hibiscus sabdariffa* using preparative reversed-phase HPLC, and the *in vitro* ACE Inhibition assay, as biological monitor model, were used for the isolation. The isolated compounds were characterized by spectroscopic methods.
Results: The anthocyanins delphinidin-3-*O*-sambubioside (**1**) and cyanidin-3-*O*-sambubioside (**2**) were isolated by bioassay-guided purification. These compounds showed IC_{50} values (84.5 and 68.4 μg/mL, respectively), which are similar to those obtained by related flavonoid glycosides. Kinetic determinations suggested that these compounds inhibit the enzyme activity by competing with the substrate for the active site.
Conclusions: The competitive ACE inhibitor activity of the anthocyanins **1** and **2** is reported for the first time. This activity is in good agreement with the folk medicinal use of *Hibiscus sabdariffa* calyces as antihypertensive.

1. Introduction

Hibiscus sabdariffa L. (Malvaceae) is an important medicinal plant growing in Africa, South East Asia, and Central America. In México, it is known as "jamaica" or "flor de jamaica" and it is widely used for preparing beverages with culinary and medicinal objectives. The traditional medicine use the aqueous extract of this plant as diuretic, for treating gastrointestinal disorders, liver diseases, fever, hypercholesterolemia, and hypertension (Monroy-Ortiz and Castillo-España, 2007).

Previous phytochemical studies on *Hibiscus sabdariffa* have reported the presence of phenolics, organic acids, sterols, terpenoids, polysacharides and some minerals. The phenolic content in the plant consists mainly of anthocyanins like delphinidin-3-*O*-glucoside, delphinidin-3-*O*-sambubioside, and cyanidin-3-*O*-sambubioside (Ali et al., 2005). *Hibiscus sabdariffa* extracts have demonstrated to have a broad range of therapeutic effects (Ali et al., 2005) such as hepatoprotective (Liu et al., 2006), antioxidant (Olatunde and Fakoya, 2005; Ramakrishna et al., 2008), anti-obesity (Alarcón-Aguilar et al., 2007), anticholesterol (Lin et al., 2007), anticancer (Olvera-Garcia et al., 2008), inhibition of the contractility of rat bladder and uterus (Fouda et al., 2007), antibacterial (Liu et al., 2005), and antihypertensive (Herrera-Arellano et al., 2007). Different works have shown that *Hibiscus sabdariffa* extracts reduce blood pressure in humans (Haji and Haji, 1999; Herrera-Arellano et al., 2004, 2007), and has been postulated that the hypotensive action could be ascribed to a direct vase-relaxant effect (Adegunloye et al., 1996). Another possible mechanism may be inhibition of angiotensin I converting enzyme (ACE). The latter action has been demonstrated *in vitro* with a crude hydroethanol extract of *Hibiscus sabdariffa* calyces (Jonadet et al., 1990). Ajay et al. (2007) demonstrated that HSE has a vasodilator effect in the isolated aortic rings of hypertensive rats. Recently, a clinical trial on 193 patients with

* Corresponding author. Tel.: +52 777 3297997; fax: +52 777 3297998.
E-mail address: lalvarez@ciq.uaem.mx (L. Alvarez).

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hypertension stages I and II was carried out by our research group. In this study, we demonstrated that *Hibiscus sabdariffa* extract exerts antihypertensive action through two modes of action that are complementary: diuretic (probably as an aldosterone antagonist) and ACE inhibitor (Herrera-Arellano et al., 2007). It has been claimed that the angiotensin converting enzyme (ACE) inhibitory activity of *Hibiscus sabdariffa* is probably due to flavones and anthocyanins presents (Jonadet et al., 1990; Odigie et al., 2003); however, this has not been demonstrated clearly yet. Despite the great interest on the antihypertensive properties of *Hibiscus sabdariffa*, until now, the chemical structure of the ACE inhibitor constituents of this important medicinal plant remain unknown. In this work, bioassay-guided fractionation, using the *in vitro* inhibition of ACE as monitor model, of the aqueous extract of dried calyces of *Hibiscus sabdariffa* afforded the anthocyanins delphinidin-3-*O*-sambubioside (**1**) and cyanidin-3-*O*-sambubioside (**2**), which inhibited ACE activity *in vitro*; the type of inhibition of these compounds was also characterized by kinetic studies.

2. Materials and methods

2.1. General experimental procedures

ACE from rabbit lung (EC 3.4.15.1) and N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG) were obtained from Sigma (St. Louis, MO, USA). ^{1}H and ^{13}C NMR spectra for anthocyanins were obtained on a Varian Unity NMR spectrometer with standard pulse sequences operating at 400 MHz in ^{1}H and 100 MHz in ^{13}C NMR. Proton and carbon chemical shifts were referenced to the residual proton and carbon solvent resonance (CD_3OD-d_4 at 3.31 and 49.15 ppm respectively). HPLC analysis were carried out on a Waters 2695 Separations Module System equipped with a Waters 717 plus Autosampler and 996 Photodiode Array Detector (Waters Co., Milford, MA). Electronic absorption spectra were recorder at 25 °C on a Hewlett-Packard 8452A diode-array spectrometer.

2.2. Plant material

Hibiscus sabdariffa L. calyces were obtained from a controlled crop in Xochitepec town in Morelos State, México, in January 2001. A voucher specimen was prepared and deposited at the IMSS herbarium for reference (#14,290) and identified by Abigail Aguilar.

2.3. Extraction and isolation

Calyces were selected and dried under dark conditions at room temperature. Dry calyces of *Hibiscus sabdariffa* (500 g) were extracted with water (250 mL) at 60 °C during 2 h. The aqueous extract was freeze-dried. The powdered aqueous extract (12.5 g) was macerated with methanol (3 × 100 mL) to give, after removal the solvent, an anthocyanin-rich fraction (HSFM, 4.21 g), this fraction was subjected to preparative reversed-phase HPLC analysis, using a Waters® XTerraPrep RP18 semipreparative column (7.8 mm × 50 mm; 5 μm particle size) and a tertiary eluent system consisting of 1.1% TFA in water (solvent A), methanol (solvent B) and acetonitrile (solvent C) at a flow rate of 1 mL/min. Isocratic profile of 80% A, 10% B and 10% C, was maintained during 10 min and detection was carried out at 520 nm. 150 μL were repetitively injected to obtain **1** (7 mg, t_R 3.6 min, 7.57×10^{-3}% yield) and **2** (5 mg, t_R 5.9 min, 4.63×10^{-3}% yield). These compounds were characterized as delphinidin-3-*O*-sambubioside (**1**) and cyanidin-3-*O*-sambubioside (**2**) by agreement of the spectral data with those reported in the literature (Du et al., 2004).

2.4. ACE inhibition assay

The evaluation of the *in vitro* ACE inhibitory activity was carried out quantifying the hydrolysis of FAPGG by ACE, using the method described by Herrera-Arellano et al. (2007). Briefly, a final volume of 730 μL of which 530 μL correspond to the substrate solution (FAPGG 3 mM in reaction buffer), and 200 μL to the reaction buffer (HEPES 25 mM, NaCl 293 mM, pH 8.3) was incubated during 3 min at 37 °C. The reaction was started by adding 20 μL of ACE solution (0.05 U/mL) to the test reaction; samples were incubated during 60 min. The reaction was stopped by adding 80 μL of 5% trifluoroacetic acid solution and the samples were centrifuged at 8952.004 × *g* for 5 min at room temperature. In the bioassay of ACE inhibition, 200 μL of buffer reaction were substituted by the same volume of extract, fraction or isolated compounds solution, in order to adjust the inhibitor concentration at 200 μg/mL.

For FAPGG kinetic hydrolysis determination in the presence of the inhibitors, solutions of different concentrations of FAPGG (0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1 mM) dissolved in HEPES reaction buffer were prepared, and 530 μL of each solution were added to 200 μL of the inhibitor in order to adjust the inhibitor concentration at 200 μg/mL.

The enzymatic activity was calculated by quantifying the decreasing of FAPGG concentration by recording the decrease absorbance at 345 nm using reversed-phase HPLC (GRACE® Altima HP C18 HL column (53 mm × 7 mm, 3 μm)) with isocratic solvent system consisting of acetonitrile–1.1% TFA in water (75:25, v/v) at a flow rate of 1.5 mL/min. FAPGG displayed a retention time of 8.67 min. The enzyme inhibition was calculated by comparing the enzymatic activity with, and without inhibitor using the following equation: % IACE = $I \times 100$; where: $I = 1 - a$ and a = activity with inhibitor/activity without inhibitor (Segel, 1975). The therapeutic drug lisinopril was used as a reference ACE inhibitor. The inhibitory concentration 50 (IC_{50}) was calculated from a linearized dose–response curve by plotting log V_0 vs. substrate concentration. The concentrations evaluated were 12.5, 25, 50 and 75 mg/mL for the aqueous extract and 50, 100, 200, 300 and 400 μg/mL for HSFM, and pure compounds **1** and **2**. The determinations were carried out in triplicate.

2.5. Kinetic calculations

The kinetic parameters were calculated by adjusting curves to the Michaelis–Menten equation: $V_0 = (V_{max} \cdot [S])/(K_M + [S])$. The inhibition type and the inhibitory constants were calculated from the double reciprocal plot, by using the equation: $m_i = m((1 + [I])/K_i)$, where m_i = slope of lineal plot from inhibited reaction, m = slope of lineal plot from reaction without inhibitor, [I] = milimolar concentration of inhibitor, and K_i = inhibitory constant.

2.6. Statistical analysis

Statistical analysis was performed by Student's *t*-test, error probabilities of $p < 0.05$ were considered to be significant. All results are presented as the mean ± standard deviation of three independent experiments.

3. Results and discussion

From the active aqueous extract of *Hibiscus sabdariffa*, an anthocyanin-rich fraction (HSFM) was obtained; this fraction inhibited the ACE activity in a dose-dependent manner with IC_{50} = 91.2 μg//mL. RP-HPLC purification of this fraction afforded two pure anthocyanins which were characterized as delphinidin-3-*O*-sambubioside (**1**) and cyanidin-3-*O*-sambubioside (**2**). The structures of these compounds were determined on the basis of

Table 1
IC_{50} values for the aqueous extract, fraction HSFM and pure compounds **1** and **2** isolated from *Hibiscus sabdariffa*.

Inhibitor	IC_{50} (μg/mL)	IC_{50} (μM)
Aqueous extract	40.04[a]	
HSFM	91.22 ± 5.74	
1	84.55 ± 2.21	141.61 ± 0.003
2	68.41 ± 2.87	117.75 ± 0.004
Lisinopril	1.2×10^{-4} ± 1.2	1.8 ± 0.002

[a] Calculated from its K_i value by using the Cheng–Prusoff equation (Cheng, 2002).

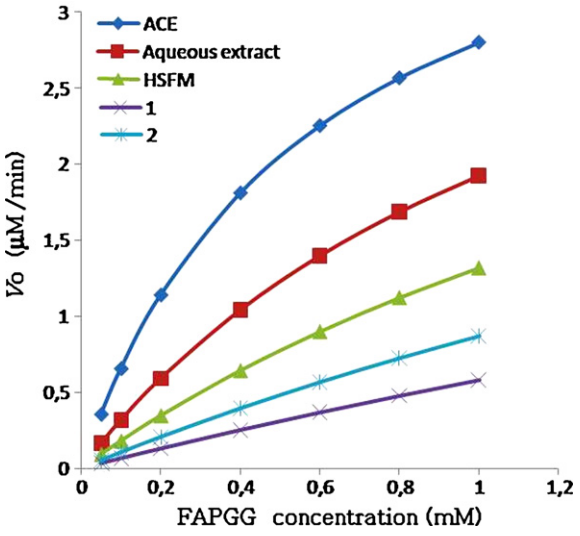

Fig. 1. Initial velocities for the inhibition of the ACE activity by 200 μg/mL of aqueous extract, fraction HSFM, delphinidin-3-*O*-sambubioside (**1**) and cyanidin-3-*O*-sambiubioside (**2**) vs. substrate concentration.

the spectral data (^{1}H NMR and ^{13}C NMR) identical with those previously described (Du et al., 2004), and which were obtained at the same conditions.

Isolated compounds inhibited the ACE activity in a dose-dependent manner, and the IC_{50} values are showed in Table 1. In order to study the type of inhibition of the ACE activity, kinetic studies were performed using extract, fractions and pure products obtained from the bioassay-guided fractionation (Table 2). Fig. 1 shows the kinetics of the ACE activity without inhibitor, and in the presence of a known concentration of the aqueous extract (50 mg/mL), HSFM (200 μg/mL), delphinidin-3-*O*-sambubioside (**1**, 200 μg/mL) and cyanidin-3-*O*-sambiubioside (**2**, 200 μg/mL).

The kinetic parameters obtained from these curves are shown in Table 2. ACE showed a Michaelis–Menten mechanism. The maximum rate of substrate hydrolysis (V_{max}) and the apparent Michaelis constant (K_{Mapp}) were determined to characterize the kind of inhibition exerted by extract, fraction and pure products isolated (Segel, 1975). In general, the parameter V_{max} was not modified significantly using the extract, fraction and pure compounds, which allow us to ponder a competitive enzyme inhibition in all the products obtained from *Hibiscus sabdariffa* calyces. The dissociation constant for the binding of inhibitor to the free enzime (K_i) was calculated for the ACE inhibition by the aqueous extract, HSFM, delphinidin-3-*O*-sambubioside (**1**) and cyanidin-3-*O*-sambubioside (**2**) (Table 2). K_i values showed that as advancing in the purification, the effectiveness of the inhibitor activity was increased. Compared with the aqueous extract (K_i = 39.871 mg/mL), HSFM (K_i = 0.065 mg/mL) was 613 times more effective. Both antocyanins were more effective than the fraction HSFM, being delphinidin-3-*O*-sambubioside (**1**) more effective (K_i = 31.9 μM) than cyanidin-3-*O*-sambubioside (K_i = 56.9 μM) which could be related with the fact that **1** has one more hydroxyl group than **2**. However, lisinopril ($K_i = 2.8 \times 10^{-4}$ μM) was more effective than **1** and **2**.

There are various reports that demonstrated that flavonoids inhibit the ACE (Kameda et al., 1987; Wagner et al., 1991; Wagner and Elbl, 1992; Lacaille-Dubois et al., 2001; Häckl et al., 2002; Kang et al., 2003; Actis-Goretta et al., 2003; Kiss et al., 2004; Oh et al., 2004; Loizzo et al., 2007).

The IC_{50} values of anthocyanins **1** (141.61 ± 0.003 μM) and **2** (117.75 ± 0.004 μM) are similar than those obtained by the related flavonol glycosides and their gallates reported by Loizzo et al. (2007), Kiss et al. (2004) and Oh et al. (2004), such as apigenin (280 μM), luteolin (290 μM), kaempferol-3-O-β-galactopyranoside (260 μM), luteolin-7-O-β-glucopyranoside (280 μM), quercetin glucuronide (200 μM), quercetin 3-O-(6″-galloyl)-galactoside (160 μM), and quercetin-3-O-α-(6‴-caffeoylglucosyl-β-1,2-rhamnoside) (158.9 μM), among others. By the other hand, the inhibition of ACE by tannins, especially by oligomeric procyanidins is well established (Ottaviani et al., 2006; Actis-Goretta et al., 2003; Lacaille-Dubois et al., 2001; Wagner and Elbl, 1992). The dissociation constant (K_i) calculated for anthocyanins **1** (K_i = 31.9 μM) and **2** (K_i = 56.9 μM) are in accordance with those reported for the flavan-3-ol epichatechin (K_i = 828 μM), and its hexamer (K_i = 4.7 μM) (Actis-Goretta et al., 2003).

ACE is a zinc-containing peptidyl dipeptide hydrolase. The active site of ACE is known to consist of three parts; a carboxylate binding functionality such as the guanidinium group of Arg, a pocket that accommodates a hydrophobic side chain of C-terminal amino acid residues, and a zinc ion. Some authors suggest that the activity of flavonoids and other polyphenols is due to the formation of chelate complexes with the zinc atom within the active centre of zinc-dependent metallopeptidases (Chen et al., 1992). Possibly it also results from the formation of hydrogen bridges between the inhibitor and amino acids near at the active site (Bormann and Melzig, 2000; Lacaille-Dubois et al., 2001).

The inhibition of ACE by anthocyanins **1** and **2**, may be probably due to the rigid planar structure of the molecule and the presence of *ortho*-dihydroxylation on the aromatic ring (Parellada and Suarez, 1998), besides appropriate hydroxylation, also a planar structure is indispensable for the metallopeptidases inhibition.

Table 2
Kinetic parameters of the ACE inhibitor activity of extract, fraction HSFM and pure compounds **1** and **2** isolated from *Hibiscus sabdariffa*.

Compound	Conc. (mg/mL)	K_M[a] (mM)	V_{max} (μM/min)	K_i (mg/mL)	K_i (μM)	IACE[b] (%)
No inhibitor		0.5751	4.4111			
Aqueous extract	50	1.2998	4.4263	39.871	–	31.36 ± 0.19
HSFM	0.2	2.3400	4.4089	0.065	–	52.01 ± 6.45
1	0.2	6.6137	4.4188	0.019	31.9	79.27 ± 9.81
2	0.2	4.0520	4.4088	0.032	56.9	68.7 ± 3.61
Lisinopril	0.001	5.9900	4.4111	1.2×10^{-7}	2.8×10^{-4}	87.18 ± 1.16

[a] For inhibition of ACE by inhibitors K_M is defined as K_{Mapp} since it is affected by the factor $(1 + [I])/K_i$ (Segel, 1975).
[b] Values are means ± SD from three separate experiments.

Anthocyanins are the major constituents in *Hibiscus sabdariffa* (Kong et al., 2003) and are used as food colouring agents (Esselen and Sammy, 1975), and diverse pharmacological investigations have demonstrated their antioxidant activity, protective effects against induced hepatic toxicity in rats, DNA damage and peroxidase activity in human's blood (Ali et al., 2005). Also, it was demonstrated that induce apoptosis in cancer human cells (Lo et al., 2007; Chang et al., 2006, 2005; Hou et al., 2005; Lin et al., 2005).

In summary, in this study, we demonstrated that the ACE inhibitor compounds presents in the calyces of *Hibiscus sabdariffa* are the two most abundant anthocyanins, delphinidin-3-*O*-sambubioside (**1**) and cyanidin-3-*O*-sambubioside (**2**). In addition, the kinetic analysis of the results suggests that these compounds inhibit the enzyme activity by competing with the active site. This is the first report on the competitive ACE inhibitor activity of the aqueous extract of *Hibiscus sabdariffa* and its principal constituents **1** and **2**.

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