Spermidine Derivatives in Lulo (Solanum quitoense Lam.) Fruit: Sensory (Taste) versus Biofunctional (ACE-Inhibition) Properties

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Supporting Information

ABSTRACT: The bitterness in lulo (Solanum quitoense Lam.) fruit is increased during processing (juicing or drying). To identify the bitter-active compounds, the ethanolic fruit pulp extract was subjected to RP-18 solid-phase extraction, and then sensory-guided fractionated by HPLC. Two spermidine derivatives, \(N^0,N^0,N^0\)-tris(dihydrocaffeoyl)spermidine and \(N^0,N^0,N^0\)-bis(dihydrocaffeoyl)spermidine, were isolated and their structures confirmed by analysis of their HPLC-ESI/MS and \(^1\)H and \(^13\)C NMR data. The \(N^0,N^0,N^0\)-tris(dihydrocaffeoyl)spermidine was synthesized and used as an authentic sample to unequivocally confirm the structure of this compound and to quantitate it in both fresh and dried fruit. In silico analyses demonstrated that spermidine derivatives identified in lulo pulp exhibited a strong ACE-1 (angiotensin I-converting enzyme) inhibitory activity. Subsequently, these results were confirmed by in vitro analyses and showed the potential use of lulo fruit pulp as an ingredient of functional foods related to the prevention of blood hypertension.

KEYWORDS: Solanaceae, ACE-inhibitory activity, bitterness, antihypertensive activity, molecular docking

INTRODUCTION

Lulo (Solanum quitoense L., Solanaceae) is a tropical fruit characterized by its refreshing and intense flavor,¹ which makes it a promising fruit to access new markets. Drying of this fruit has been used as a strategy to extend its postharvest shelf life, preserving its nutritional and functional properties.²,³ However, with time an undesirable bitter note is increasingly perceived in either the juice or dried fruit. This detrimental change in the sensory qualities is a limiting factor for the acceptance by consumers, who usually associate the bitter taste with sensory-guided fractionation and subsequent spectroscopic analyses, in order to estimate the influence of these compounds in fresh and dried fruits. After identifying those compounds, it was found that some structure-related compounds had been reported as hypertensive principles of oriental medicine “jikoppi”, which is clinically effective for hypertension.¹² Thus, in silico and in vitro analyses were hypertension has particular relevance to modern medicine, as it is a key symptom of metabolic syndromes, themselves important risk factors for the development of cardiovascular diseases. Studies have suggested that angiotensin I-converting enzyme (ACE) is an enzyme associated with the blood pressure (BP) regulating renin–angiotensin system (RAS); specifically, it catalyzes both the production of the vasoconstricting peptide angiotensin II (AngII) and the inactivation of the vasodilator bradykinin.⁹ Thus, inhibition of ACE may exert an antihypertensive effect, and potent synthetic inhibitors of ACE are used in the treatment of hypertension.¹⁰ Additionally, significant correlations between increased ACE inhibition and bitterness have been found for dipeptides using both observed and QSAR-predicted values.¹¹

Hypertension has particular relevance to modern medicine, as it is a key symptom of metabolic syndromes, themselves important risk factors for the development of cardiovascular diseases. Studies have suggested that angiotensin I-converting enzyme (ACE) is an enzyme associated with the blood pressure (BP) regulating renin–angiotensin system (RAS); specifically, it catalyzes both the production of the vasoconstricting peptide angiotensin II (AngII) and the inactivation of the vasodilator bradykinin.⁹ Thus, inhibition of ACE may exert an antihypertensive effect, and potent synthetic inhibitors of ACE are used in the treatment of hypertension.¹⁰ Additionally, significant correlations between increased ACE inhibition and bitterness have been found for dipeptides using both observed and QSAR-predicted values.¹¹

The aim of this study was to identify bitter-active compounds in S. quitoense fruit by using sensory bioguided fractionation and subsequent spectroscopic analyses, in order to estimate the influence of these compounds in fresh and dried fruits. After identifying those compounds, it was found that some structure-related compounds had been reported as hypertensive principles of oriental medicine “jikoppi”, which is clinically effective for hypertension.¹² Thus, in silico and in vitro analyses were

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performed to evaluate the ACE-I (angiotensin I-converting enzyme) inhibitory activity of lulo bitter compounds.

**MATERIALS AND METHODS**

**Chemicals.** The ethanol used for the preparation of fruit extract was food grade (200 proof, Fisher Scientific, Fair Lawn, NJ, USA). Formic acid, ethanol, methanol, and acetonitrile were HPLC-grade solvents obtained from Fisher Scientific (Fair Lawn, NJ, USA). Distilled and deionized water used for HPLC separation were purified through a Barnstead Nanopure Diamond water purification system (Thermo Scientific, Dubuque, IA, USA). Methanol-d₄ (99.8% enrichment), caffeine, and hexane were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). THF, DME, DCC, chloroform, methanol, dichloromethane, NaHCO₃ and the other reagents used in the synthesis of compound 1 were purchased from Tokyo Chemical Industry (Tokyo, Japan).

**General.** ¹H and ¹³C NMR spectra of compound 3 and intermediates of the synthesis of compound 1 were recorded on a Bruker DRX500 (500 MHz for ¹H and 125 MHz for ¹³C). A Bruker Avance-700 spectrometer with TXI cryoprobe was used for ¹H and ¹³C NMR spectra of compounds 1 and 2. Data processing was performed using MestReNova 8.1 (Santiago de Compostela, Spain) and Top spin 2.1 (Rheinstetten, Germany) softwares, respectively. NMR spectra recorded in CDCl₃ were referenced to TMS signal (δ 0.0) for ¹H and the solvent signal (δ 77.0) for ¹³C; for the spectra recorded in CD₂OD, the residual nondeuterated solvent signal at δH 3.30 and the solvent signal at δC 49.00 were set as the internal reference. HRFABMS spectra of synthetic compounds 1, 7, and 9 were obtained on a JEOL JMS-700 spectrometer with 3-nitrobenzyl alcohol as the matrix. HPLC-MS of synthetic compounds 1 and 9 were recorded on a Shimadzu LC-MS 2020 (Tokyo, Japan) spectrometer in a DUIS ionization mode. For preparative purposes a Shimadzu HPLC apparatus was used, consisting of a binary pumping system (LC-10 ADvp), a degasser (DGU-14A), an autosampler (SIL-10vp), a fraction collector (Shimadzu FRC-10A), and a variable-wavelength UV-vis detector at 254 and 280 nm (Shimadzu SPD-M10A).

**Plant Material.** Fresh lulo (Solanum quitoense Lam.) fruits were obtained from a local market in Bogota, Colombia, and processed immediately upon arrival at the laboratory. Ripe fruits were classified as “extra” quality, and their ripening stage was selected according to their 100% orange peel color, equal to #5 in the color scale (ripening scale defined by the color changes occurring during this process, from #0, equal to fruit setting, to #5, equal to ripe fruit to be consumed).¹³ The pH value of fruit pulp was 3.10 ± 0.05, and soluble solids content (SS) was 7.8 ± 0.2 °Brix (data are given as average ± standard deviation, n = 5).

**Preparation and Fractionation of the Pulp Fruit Extract.** Fresh lulo fruits without peel (5 kg) were cut into pieces (2 cm × 2 cm) and freeze-dried in a ViraTis Genesis lyophilizer (Gardiner, New York, NJ, USA) to get 500 g of dried fruit. Then lyophilized fruits were extracted with ethanol (100 g, 3 × 300 mL) at 18 °C for 24 h with constant stirring. The solvent was removed under vacuum, and the residue afforded a crude extract after freeze-drying. This extract (50 g) was fractionated by SPE (solid phase extraction, 10 g C₁₈ cartridges, Supelco, Bellefonte, PA, USA) in portions of 1 g dissolved in 5 mL of distilled water. Three fractions were collected in each separation with 200 mL of the following eluent solutions: distilled water, ethanol, and hexane, to obtain three fractions, F₁ (43 g), F₂ (2.1 g), and F₃ (4.0 g), respectively. The solvents of each fraction were removed under vacuum distillation and the residues dissolved in distilled water and freeze-dried twice, for subsequent sensory analyses.

**HPLC Fractionation.** The bitter fraction F₂ (500 mg) was dissolved in a mixture of ethanol—water (50:50 v/v) and fractionated by HPLC using a semipreparative Restek Ultra Aqueous C₁₈ column (250 × 10 mm i.d.; particle size 5 μm; State College, PA, USA). The injection volume was 50 μL. The solvent system was a mixture of water/formic acid (99.9:0.1, v/v, solvent A) and ethanol (solvent B), and the flow rate was 3.5 mL/min. A linear gradient from 10 to 50% B at 0–15 min, 50% B during 15–20 min, 50 to 100% B at 20–30 min, 100% B 30–35 min, 10% B at 35–40 min was used. Sixteen fractions (F₂.1–F₂.16, 2 min each) were collected at λ = 254 nm, the solvent was removed under vacuum, and the samples were lyophilized (x2) for sensory and LC-ESI-MS analyses. Then, bitter fractions F₂.4 (22.8 mg), F₂.5 (16.4 mg), and F₂.6 (17.6 mg) were analyzed in a second chromatographic column, using a Zorbax Bonus-RP C₁₈ column (150 × 2 mm i.d.; 5 μm). Four compounds (1–4, Figure 1) were purified by preparative HPLC. The separations were carried out as follows: the injection volume was 50 μL, the column temperature was 25 °C, binary mobile system A was 0.1% formic acid aqueous solution, pH 3.0, and B was 100% acetonitrile, and the flow rate was 0.5 mL/min. The gradient was started with 5% B (0–2 min), then increased to 40% B (2–20 min), increased to 90% B (20–25 min), then held at 90% B (25–27 min), and then decreased to 5% B (27–32 min). From F₂.6 was obtained pure compound 1 (12 mg); from F₂.4 were obtained compounds 2 (10 mg) and 3 (8 mg); and from F₂.5 was obtained compound 4 (6 mg). Purified compounds were identified on the basis of their LC-ESI-MS and NMR spectra. The solvent was removed in all the fractions, and then the samples were freeze-dried twice and reconstituted in distilled water for further sensory analysis.

**LC-MS Analyses.** LC-MS analyses of fractions and pure compounds were performed using a Shimadzu LCMS-2010 system (Shimadzu, Tokyo, Japan) equipped with a UV/vis detector (SPD-10A) and two pumps (LC-10 ADvp) coupled online with an MS-2010 mass spectrometer. UV and MS data were acquired and processed using Shimadzu LCMS Solution software. The equipment also included an online DGU-14A degasser and a Rheodyne injection valve with a 5 μL loop. The column and gradient used were identical to those described above for HPLC. The electrospray ionization (ESI) probe was operated simultaneously in the positive and negative modes: CDL and block at 250 °C; flow gas (N₂) at 1.5 L/min; CDL voltage, 1.8 kV; Q array voltage RF 150 V; detector voltage, 1.5 kV; and scan range m/z 100–1000 u.

Figure 1. Chemical structures of the bitter compounds identified in Solanum quitoense fruit pulp. Compound 3 was isolated in the formic acid salt form.
For the accurate mass analysis of pure compounds (1 and 2) a Waters 2D UPLC Acquity iClass coupled with a Xevo G2 QTOF mass spectrometer (Waters, Milford, MA, USA) was used. Accurate mass acquisition of the ion of interest was performed by chromatography injection using a dual electrospray ion source. Reserpine (0.5 mg/L) was used as the locksspray injecting at 20 μL/min. Samples of 2 μL of each compound were injected on an ACQUITY UPLC BEH C18 1.7 μm column (2.1 mm × 50 mm; Waters) maintained at a temperature of 25 °C. The mobile phase was maintained at a flow rate of 0.3 mL/min using a binary solvent system of 0.1% formic acid in water (A) and methanol (B). The elution gradient started at 10% B (0–1 min), was linearly increased to 50% B (1–6 min), then increased to total 100% B (6–8 min), and finally returned to the initial conditions (10–15 min). Mass spectrometric ionization conditions were as follows: desolvation temperature of 350 °C; source temperature of 110 °C; capillary voltage of 2.0 kV. Compounds were analyzed in both positive and negative sensitivity scan mode, and the scan range was 50–1000 Da. Data processing was performed using Waters MassLynx 4.1 software.

Sensory Analyses. An experienced sensory panel consisting of eight judges (ages 22–45, 3 males and 5 females), from the Department of Food Science and Nutrition, St. Paul, MN, evaluated the taste and astringency attributes of S. quitoense fruit HPLC fractions. Prior to analysis, all panelists were trained with reference solutions for taste and astringency attributes of interest (10 for taste analysis, prior to sensory testing, were liberated from solvent fractions treated by the above protocol are free of solvents and suitable for sensory analysis. The Ethics Committee, University of Minnesota, granted IRB approval of the sensory evaluation protocol (IRB #1505E70948).

Spectroscopic Data. In this section, the spectral data of compounds 1–4 are reported. Numbering of compounds refers to Figure 1.

\[ \begin{align*}
N^1,N^4,N^8-Tris(dihydracaffeoyl)spermidine (1): & \text{ white solid. MS/MS TOF: } m/z 638.2188 ([M + H]^-); \\
& \text{MS/MS TOF: } m/z 387.1884 ([M + H]^-); \\
& \text{NMR (CDCl}_3, 500 MHz) \text{, see Table 1.} \\
\end{align*} \]

**Table 1.** 1H and 13C NMR Spectral Data for Bitter Compounds 1 and 3 from Lulo (S. quitoense Lam.) Fruit (CD_3)OD, δ in ppm, J in Hz

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**Figure 1.**

Obtention of N 1,N4,N8-Tris[3-(3,4-methylenedioxyphenyl)propionyl]spermidine (9). To a solution of 3-(3,4-methylenedioxyphenyl)propionic acid (8) (900 mg, 4.63 mmol) and N-hydroxysuccinimide (6) (534 mg, 4.63 mmol) in dry THF (6.8 mL) and DMF (2.3 mL) was added DCC (1.01 g, 4.87 mmol) at 0 °C, and the mixture was stirred without cooling for 12 h. The precipitated urea was filtered off, and the filtrate was concentrated to dryness under reduced pressure. The residue was chromatographed over silica gel with CHCl3–MeOH (90:1) to give the succimidyl ester (7) (785 mg, 2.70 mmol, 58%) as a white solid. Mp: 112–113 °C. UV (MeOH): λ_{max} = 206, 235, 287.5 nm. 1H NMR (CDCl3, 500 MHz): δ: 6.75 (d, J = 7.9 Hz, 1H), 6.72 (d, J = 1.7 Hz, 1H), 6.68 (dd, J = 7.9, 1.7 Hz, 1H), 5.93 (s, 2H), 2.98 (t, J = 7.6 Hz, 2H), 2.87 (t, J = 7.6 Hz, 2H), 2.84 (br, 4H). 13C NMR (CDCl3, 125 MHz): δ: 169.1, 167.8, 147.8, 146.3, 132.8, 121.2, 108.8, 108.4, 101.0, 30.3, 30.2, 25.6. HRFABMS: m/z 529.0848 [M + H]^+ (calcld for C_{22}H_{28}O_{14}N_4, 529.0821).
MH+ \delta: 172.4, 172.1, 171.6, 147.4, 147.3, 147.2, 145.7, 145.7, 145.6, 134.6, 134.4, 120.9, 120.9, 120.9, 108.7, 108.6, 108.5, 108.0, 108.0, 100.6, 100.6, 100.5, 47.2, 45.5, 45.2, 42.3, 38.4, 38.4, 38.3, 38.2, 38.1, 36.6, 34.9, 34.8, 31.3, 31.3, 31.2, 31.1, 29.1, 27.2, 26.9, 26.2, 25.8, 24.5, 24.3. HRFABMS: \mathrm{m/ z} = 551 \ [ \mathrm{M + H} ]^+ \ \text{(calcd for C37H44N3O9 551.3078). LC-MS (ESI+):} \\
\mathrm{m/ z} = 638 \ [ \mathrm{M + Na} ]^+ \ [ \mathrm{M + Na} ]^+ \ \text{(calcd for C34H44N3O9 638.3078). LC-MS (ESI-):} \\
\mathrm{m/ z} = 672 \ [ \mathrm{M - H} ]^- \ \text{was observed at 10.6 min (analyzed in the same conditions as described above). Forero et al. \cite{1})]. Standard additions at five levels of compound 1 (7 to 400 mg/L for fresh fruit and 0 to 4000 mg/L for dried fruits) were used to establish the regression equation used to quantify lulo samples that showed good linearity with regression coefficients of 0.9840 and 0.9975, for fresh and dried fruits, respectively. Compound 1 was detected in all the samples by monitoring its pseudomolecular ion at \mathrm{m/z} 638 in positive SIM mode. All samples were analyzed in triplicate. The analytical parameters determined were as follows: limits of detection (LOD) and quantitation (LOQ) from the calibration graph mentioned above, according to International Conference on Harmonization (ICH) guidelines. \cite{20} Determination of the LOD and LOQ were based on the standard deviation of the intersection of the analytical curve (\sigma) and on the slope of the curve (S). The LOD was expressed as 3.3 (%/S) and LOQ as 10 times (\sigma/S).

In Silico Analyses of ACE-1 Inhibitory Activity. Molecular docking experiments were performed using Autodock/Vina (1.1.2) package under Linux in a Phyton 2.7 environment, using the AMBER force field. \cite{21} All calculations were performed on a workstation equipped with an Intel Xeon processor (32 cores) and 64 GB of RAM. The protein and ligand preparations were carried out as follows: the X-ray crystallographic structure of the enzyme (angiotensin-converting enzyme ACE-1) was obtained from the Protein Data Bank (PDB code: 1IO8A) at a resolution of 2.0 Å. Water molecules and ligands were removed from the protein molecule along with the chains except chain A. Addition of hydrogen atoms to the protein was performed, and the energy minimization was made with the CHARMM force field by using the conjugate gradient method with an RMS gradient of 0.01 kcal/Å mol on Discovery Studio 2.5 software. \cite{22} Structural information of the ligands, compounds 1, 2, 3, and the structurally related N1-(dihydrocaffeoyl)spiropiperidine (10) was prepared by using a Monte Carlo randomized conformational search without any geometrical restrictions, employing the Merck Molecular Force Field (MMFF94) included in the SPARTAN software with a limit of 500 conformers. \cite{23} The lowest energetically stable conformers within a 6 kcal/mol energy range were geometrically optimized using semiempirical AM1 parametrization implemented in the software package. The minimized protein and ligands were saved in PDB format for further docking analysis. The Audock/Vina algorithm on the ACE–ligand complexation was validated through a redocking protocol using the co-crystallized lisinopril into the active site of ACE. The root mean square deviation (RMSD) in Å between resulted poses was calculated for validation purposes.

The docking experiments on test enzyme were carried out between the energy-minimized ligand into the active site through a cube at the geometrical center of the native ligand present in the evaluated PDB structure, with dimensions of 22 × 22 × 22 Å, covering the ligand binding site with a grid point spacing of 0.375 Å. Flexible residues were thus selected within 4 Å of this geometrical center at active site using the Autodock/Vina plugin. The docking poses were ranked according to their docking scores (as free energy of binding), and both ranked list of docked ligands and their corresponding binding pose scores were exported and viewed using Pymol (1.3.2x). \cite{24} All of the analyses were performed three times, and lisinopril was used as positive control. Finally, the resulting docking information (docked enzyme–ligand complex, files in .pdbqt format) was imported into the Inte:ligandLigandScout software package, and the analysis of the binding site was then performed in order to create the 2D pharmacophore models.

In Vitro Measurement of ACE Inhibition. The ACE inhibitory activity of compound 1 (0.001–21 ppm), lulo fruit pulp, and dried lulo
fruit (spray-dried and freeze-dried) was determined using an ACE inhibitory assay kit (ACE kit-WST, Dojindo Laboratories, Kumamoto, Japan) in duplicate. The assay was carried out according to the protocol provided by the manufacturer. The absorbance of the reactions was measured on a Bio-Rad iMark Microplate Reader at λ 450 nm. The ACE inhibitory activity of the samples was calculated using the formula given in the protocol.22 Blank experiments were performed in the same conditions, one without sample, and the other without enzyme. The concentration of the ACE inhibitor required to inhibit 50% of ACE activity under the above assay conditions was defined as the IC_{50}. Lisinopril was used as positive control (5–100 ppm).

**Statistical Analysis.** Using Statgraphics Plus 5.1 software (StatPoint, Inc., Herndon, VA), analysis of variance (ANOVA) and Tukey’s tests were performed to identify differences between data of quantitation of compound 1. Differences at probability level $P \leq 0.05$ were considered significant.

## RESULTS AND DISCUSSION

**Identification of Taste-Active Compounds.** As a first step in the analytical isolation procedure, the solvent extraction of the bitter-active compounds from lulo fruit was evaluated. The isolates from three solvent mixtures consisting of ethanol–water (50%, 70%, and 100%) were evaluated by the sensory panel. The extract obtained with 100% ethanol was chosen as the most bitter and subjected to further fractionation. Further fractionation of this extract by SPE yielded three fractions: F1 eluted with water, and it was described by the panelists as sour (acid and sugar enriched fraction); F2 eluted with 100% ethanol was described as astringent and bitter; and F3 eluted with hexane did not exhibited any special taste (carotenoid-rich fraction). The F2 SPE fraction was subsequently fractionated by HPLC to obtain 16 fractions (F2.1–2.16) and then each fraction evaluated by the sensory panel. The taste and astringency profile is shown in Figure 3; this graph can be considered as the taste/astringency fingerprint of the lulo fruit extract. In general, it can be stated that the lulo fruit extract is considered as the taste/astringency pro

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**Figure 3.** HPLC chromatogram ($\lambda = 254$ nm) and taste intensity profile of F2 fraction of lulo fruit extract (Restek Ultra Aqueous C18 column).

Bitter fractions F2.4, F2.5, and F2.6 were further purified by HPLC to get compounds 1–4 (Figure 1). Several stationary phases were tested, and the best resolution was obtained with the Bonus RP-18 column. F2.12 and F2.15 (non UV active) were not further purified because they were isolated in a tiny amount that was not enough to obtain useful NMR spectra for their structural elucidation. However, LC-MS analysis suggested the presence of saponin-type compound in F2.12 exhibiting a MW of 1194 u.

The fraction (F2.6) was fractionated by sensory-guided HPLC to get five subfractions, among which F2.6.3 contained the compound 1 (see Figure 1S). The high resolution mass spectrum of this compound showed an [M + H]$^+$ ion peak at $m/z$ 638.2158 in agreement with a molecular weight of $m/z$ 637 u, characteristic of an odd number of nitrogen atoms in the molecule. The MS/MS spectrum obtained from the ion peak at $m/z$ 638.2158 showed daughter ions at $m/z$ 474.1932 [M + H – 164]$^+$ and 293.5432 [M + H – 164 – NH$_3$]$^+$ that evidenced the presence of at least two dihydrocaffeoyl residues in this molecule, since each acyl group can be eliminated as a ketene (−164 u). The $^1$H NMR spectrum showed signals for 43 protons, some of them appearing in the sp$^3$ aromatic region, and the others as aliphatic methylenes between δ$^H$ 3.22 and 1.27 ppm (26 H). The COSY spectrum showed four spin systems, three aliphatic and the other aromatic-type. A correlation between the signals for a propyl group at δ$^H$ 1.60–1.53 with the signals at δ$^H$ 3.13–3.04 and 3.22–3.17 ppm was seen; also between the signals of butyl group at δ$^H$ 3.13–3.04 with the signals at δ$^H$ 1.40–1.27 (×2); between the signals of an ethylene group at δ$^H$ 2.56–2.50 and 2.78–2.72 ppm; and between the signals of an aromatic trisubstituted ring at δ$^H$ 6.67–6.61 (×3) and 6.53–6.49 ppm. There was a high overlap among all the $^1$H NMR signals (700 MHz), such indicating the presence of similar moieties in compound 1. From the HMBC and HMQC spectra, the following substructures were confirmed: the dihydrocaffeoyl substructure was identified for the characteristic correlations of the aromatic ring 1,2,4-trisubstituted, the correlations between one of the ethylene protons (δ$^H$ 2.78–2.72) and the aromatic carbons at δ$^C$ 119.2 and 115.4, and the correlation between the carbonyl at δ$^C$ 174.2 and the other ethylene protons at δ$^H$ 2.56–2.50; the propyl group was attached to the amide by the correlation...
between the ethylene protons at \( \delta_H 3.22 \text{−}3.17 \) and the carbonyl at \( \delta_C 174.2 \); the butyl group also was attached to the amide by the correlation between the ethylene protons at \( \delta_H 3.13 \text{−}3.04 \) and the carbonyl at \( \delta_C 174.2 \). These correlations allowed suggestion of the structure of \( N^2,N^4,N^8\text{-tris(dihydrocaffeoyl)} \text{-} \text{ spermidine} \) for compound 1.

With the aim to confirm the structure of the bitter compound 1 and quantitate it in the fresh and dried fruit, the synthesis of the reference compound was carried out according to the scheme of Figure 2, starting with \( 3\text{-}(3,4\text{-methyleneedioxyphenyl)} \text{-} \text{propionionic} \) (5) and \( N\text{-hydroxysuccinimide} \) (6) to produce the corresponding succimidyl ester (7). The succimidyl ester (7) was directly condensed with spermidine (8) to afford the \( N^1,N^4,N^8\text{-tris(3,4\text{-methyleneedioxyphenyl)}} \text{-} \text{propionyl}\text{-} \text{spermidine} \) (9). The last step of the synthesis consisted of the removal of the methyl protecting groups of the catechol moiety, yielding the desired compound 1. The identity of 1 was confirmed by coinjection of natural and synthetic compound in HPLC and also by comparison of \(^1\text{H}\) and \(^{13}\text{C}\) NMR data. However, NMR data are complex due to the presence of rotamers of this compound.

The quantitative analysis (Table 2) showed that \( N^1,N^4,N^8\text{-tris(dihydrocaffeoyl)} \text{-} \text{spermidine} \) was present in fresh fruit, and its concentration increased ca. 16-fold during the drying of the fruit by different methods. These results are in agreement with the increase in the bitterness perceived in the dried fruits. It is well-known that the polyamines spermine and spermidine play important roles in cell division, organogenesis, response to stress, and inhibition of lipid oxidation. Thus, it is possible to suggest that the amount of compound 1 is increased during the processing of lulo fruit as a response to the wounding stress. This protection mechanism has been documented in carrots (Daucus carota); when they are subjected to wounding and hyperoxia stresses, the production of caffeoylquinic acids and phenolic compounds is significantly increased (ca. 287% increase in total phenolic content).

Compound 1 was previously detected by HPLC-MS in lulo (Solanum quitoense), potato (Solanum tuberosum), and cocona (Solanum sessiliflorum). This is the first time that the purification, NMR analyses, synthesis, and sensory properties (bitterness) of compound 1 are reported. Several derivatives of spermidine and other polyamines have been mainly reported in other Solanacea species, such as potato and eggplant. These compounds play an important role in plant growth, stress, and disease resistance, and also have exhibited a promising biological activity.

From the other bitter fraction (F2.4) six subfractions were obtained, F2.4.3 and F2.4.4 being bitter active, and correspondingly to pure compounds 2 and 3, respectively (see Figure 1S). Compound 2 showed an \([M + H]^+\) ion peak at \( m/z 387.1884 \) (C\(_{17}\)H\(_{23}\)O\(_{10}\)) in the UPLC-MS spectrum. Additionally, ion fragments obtained by the loss of a hexose moiety (\( m/z 223 \)) and the subsequent loss of water (\( m/z 205 \)) were observed. The \(^1\text{H}\) NMR spectrum showed signals for two methoxyl groups at \( \delta_H 3.90 \text{ (3H, s)} \) and 3.88 (3H, s), one trans-double bond conjugated with a carbonyl group at \( \delta_H 7.74 \text{ (d, } \text{J = 15.9 Hz)} \), and 6.46 (d, J = 15.8 Hz), two aromatic protons from a tetrasubstituted ring at \( \delta_H 6.96 \text{ (2H, s)} \), and a hexose moiety with \( \beta\)-configuration in C-1’ (\( \delta_H 5.59 \text{, d, } \text{J = 8.1 Hz, 1H}) \). \(^{13}\text{C}\) NMR spectrum and HMBC experiments confirmed the presence of an \( \alpha\beta\)-unsaturated carbonyl group (\( \delta_C 166.6, 114.5, \) and 147.9) and a tetrasubstituted aromatic ring. The foregoing data indicated that compound 2 is the \( \beta\)-d-glucoside of synapic acid. The position of sugar was confirmed by the HMBC correlations of anomeric proton (\( \delta_H 5.59 \) and carbonyl (\( \delta_C 166.6 \)). Linscheid, Wendisch, and Strack reported this compound for the first time in nature. The NMR data of compound 2 were in agreement with those published by Harbaum et al. for the aglycon, and Roslund et al. for the sugar moiety. This is the first time that this compound is reported in S. quitoense as responsible for bitter taste.

Compound 3 showed a pseudomolecular ion \([M + H]^+\) at \( m/z 474 \), and in negative mode an ion fragment at \( m/z 472 \) (M – H\(^+\)), both in agreement with a molecular weight of 473 u. The \(^1\text{H}\) NMR spectral data of compound 1 and 3 resembled each other, but according to molecular weight, compound 3 had one dihydrocaffeoyl unit less. Thus, the structure of this compound was assigned as \( N^1,N^8\text{-bis(dihydrocaffeoyl)} \text{-} \text{spermidine} \) (Figure 1) because MS and \(^1\text{H}\) NMR data were in agreement with those published by Gancel et al., Parr et al., and Wu et al. Finally, compound 4 from F2.5 was tentatively identified as S-O-caffeoylquinic acid, according to what was previously reported by Gancel et al. in lulo fruit. This is one of the caffeic acid esters that predominates in Solanacea species; however, its individual bitterness has not been reported before.

**Measurement of ACE Inhibitory Activity.** Polyamines play multiple roles in cell growth, survival, and proliferation and have also been involved in stress resistance. Additionally, they are increased in cancer cells and are a target for potential chemotherapeutic agents, and their hypotensive effects have also been reported. It has been reported that fruits also contain these bioactive amines, which are formed during metabolic processes. Angiotensin-converting enzyme (ACE) is a type-I membrane-anchored dipeptidyl carboxypeptidase that is essential for blood pressure regulation and electrolyte balance.
homeostasis. Inhibitors of ACE are a first line of therapy for hypertension, heart failure, myocardial infarction, and diabetic nephropathy. Thus, molecular docking analysis is a useful chemical tool to predict bioactivity of molecules and thus better address in vitro and/or in vivo bioassays.

Performance of the AutoDock/Vina algorithm on ACE–ligand complexation was evaluated by a redocking protocol applied on the cocrystallized lisinopril into the active site of the enzyme (PDB code: 1O8A) with the aim to evaluate ACE-inhibition activity of lulo bitter-active compounds (1–3). After three runs, a mean RMSD of 1.35 Å (±0.45 Å) was therefore found. This RMSD value was accepted as validation criteria for the docking algorithm. The lowest-energy docked pose was also validated by observing molecular contacts with ACE pocket residues and comparing them with those reported in the crystal structure of the ACE–lisinopril complex. The same protocol was then applied with compounds 1–3 and 10. The results obtained after the docking of five ligands into the active site of ACE are shown in Figure 4. Compound 1 exhibited the best affinity value or binding score (−10.0 ± 0.2 kcal/mol), with even lower values than that of the positive control used, lisinopril (−9.0 ± 0.1 kcal/mol), followed by the amide-related compounds 3 (−8.7 ± 0.1 kcal/mol) and 10 (−6.4 ± 0.1 kcal/mol). 1-O-Sinapoyl β-D-glucoside (2) was also evaluated showing an affinity value of −8.0 ± 0.2 kcal/mol. The dispersion (% RSD) of the affinities values between replicates per compound was considered low (0.6 to 1.6), and it indicated good convergence in the calculation. Their 3D models of lowest-energy docked poses in the presence of ACE are shown in Figure 4a–c. The strong affinity values found for compounds 1 and 3 (−10.0 kcal/mol and −8.7 kcal/mol, respectively) can be attributed to the high protein–ligand interactions (H-bond and nonpolar interactions), as can be evidenced in the 2D and 3D representations (Figure 4f–j). The presence of aromatic rings in the structures can contribute to the nonpolar stabilization through PHE, TRP, and TYR residues principally, and the zinc ion (an important catalytic component of ACE) seems to have an important role in the ligand–protein interaction due to its facility to coordinate with the ligands.

The presence of nitrogen (which is a structural feature shared of the tested compounds to the active site through GLU384, ASP377, and ALA354 residues, and the presence of hydroxyl group in aromatic rings increases complex binding. Although lisinopril has important polar contacts by the presence of the ionizable carboxyl and amine groups, which seems to have an important role in the ligand–protein interaction due to its facility to coordinate with the ligands.

Figure 4. 3D model of lowest-energy docked pose of ACE-1 enzyme with bitter compounds from lulo fruit (a) N1,N1,N8-tris(dihydrocaffeoyl)-spermidine (1), (b) N1,N8-bis(dihydrocaffeoyl)spermidine (3), (c) N1-(dihydrocaffeoyl)spermidine (10), (d) 1-O-sinapoyl β-D-glucoside (2), and positive control (e) lisinopril (these 3D models show each bitter compound docked within the active site of the ACE-1; selected residues at active site or ACE-1 are marked in yellow sticks; remaining enzyme in green cartoon; ligands in differently colored sticks; polar interactions are indicated in magenta dashed lines labeled with distance in Å); and 2D pharmacophore models of ACE-1 enzyme with lulo bitter compounds (f) (1), (g) (3), (h) (10), ( i) (2), and (j) lisinopril (these 2D models show how each bitter compound interacts with residues of the active site of the ACE-1). The hydrogen bond features are depicted by the green (hydrogen bond donor) and red (hydrogen bond acceptor) hashed vectors. The hydrophobic and π–π aromatic interactions are depicted by the yellow spheres and the blue sphere + arrow, respectively. Negative and positive ionizable groups are depicted by the dark red and blue wedges, respectively.
bitterness and average hydrophobicity is called the Q rule, and empirical correlation between the presence or absence of only due to the presence of compound essential for the sensation of bitter taste. Additionally, the amounts of compound ACE-inhibitory activity than freeze-dried fruit (83.49 ppm). exhibited a significant relationship (QSAR) models for the use of the NMR instrumentation is also thanked.

The authors greatly appreciate Julie Peterson, for the coordination and training of the sensory panel and for her valuable comments regarding sensory analysis, and Qing Bing and Liyun Zhang from University of Minnesota for recording accurate mass analysis of pure compounds 1 and 2. Minnesota NMR for the use of the ACE-inhibitory activity in vitro assay of compound 1 and fresh and dried fruit which revealed a concentration dependent activity (Table 2). The IC50 value of compound 1 is 9.55 ppm, which was significantly smaller than that of the control lisinopril (709.36 ppm). The fruit pulp also exhibited a significant ACE-inhibitory activity. Among dried fruits, the spray-dried fruit (43.17 ppm) exhibited a higher ACE-inhibitory activity than freeze-dried fruit (83.49 ppm). The amounts of compound 1 in two dried lulo fruit samples are comparable, however the ACE-inhibitory activity was different in both of them (spray-dried fruit value is almost double the freeze-dried fruit value), such suggesting that this activity is not only due to the presence of compound 1 in the fruit. The empirical correlation between the presence or absence of bitterness and average hydrophobicity is called the Q rule, and reflects the assumption that a hydrophobic interaction is essential for the sensation of bitter taste. Additionally, the relation between bitterness and ACE inhibition has been studied for dipeptides by using quantitative structure–activity relationship (QSAR) models finding the effect of C-terminal hydrophobicity necessary for the ACE inhibition.11

By using a sensory-bioguided methodology of fractionation, four bitter compounds were identified and purified in S. quitoense fruit, as contributors to the bitterness increase during the juicing and drying of this fruit. However, it was also found by docking analyses and in vitro studies that compounds 1 and 3 are promising ligand candidates for the ACE inhibition. Thus, N1,N2,N3-tris(dihydrocaffeoyl)spemerdine and N2,N6-bis-(dihydrocaffeoyl)spemerdine were here identified for the first time as bitter-active compounds in S. quitoense fruit and also as bioactive amines, such converting the S. quitoense fruit pulp to a promising functional ingredient for foods to help in hypertension control. This finding gives high added value to S. quitoense fruits to promote their consumption because of their biofunctional properties. Bitterness of these compounds is manageable since this taste characteristic is modulated (imperceptible) in the fresh fruit.

**ASSOCIATED CONTENT**

Supporting Information
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