



Glucose-6-Phosphatase and α -Glucosidase Inhibitors from *Smilax moranensis* Roots Identified by Affinity-Directed Fractionation

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Abstract

The ethanol/water-soluble extracts of the roots of *Smilax moranensis* M. Martens & Galeotti, Smilacaceae, which have been appreciated since pre-Hispanic times and traditionally used to treat type 2 diabetes in Mexico, were fractionated by the application of the affinity-directed method to identify glucose-6-phosphatase and α -glucosidase inhibitors. Mass spectrometry was used to identify the inhibitor as free ligands after released from the enzymatic complex by denaturing acidic conditions. The affinity study led to the identification of chlorogenic acid as a glucose-6-phosphatase inhibitor, which is the most abundant metabolite present in the analyzed crude drug. In addition, the affinity studies led to the identification of a flavonolignan glycoside as an α -glucosidase inhibitor. *In silico* analysis with α -glucosidase MAL12 from the yeast *Saccharomyces cerevisiae* allowed to determine the best conformational rearrangement for the α -glucosidase inhibitors.

Keywords Diabetes · Enzyme inhibitors · Free radical scavenger · Hypoglycemic · Molecular docking · Polyphenols

Introduction

In Mexico, medicinal plants have traditionally played an important role (Mata-Torres et al. 2020); besides, the renewed worldwide interest in the use of herbal medicines under the belief of their lack of toxicity and their low cost has led to the use of phytopharmaceuticals for the treatment of hyperglycemia. However, very few traditional medicinal plants have been studied to verify their hypoglycemic properties, toxicity, and abundance to ensure a safe source of the medicinal plants. Time-consuming processes for the purification of bioactive natural products limit the performance of a fast and efficient way to identify bioactive molecules in crude extracts, making the isolation of active metabolites laborious. Consequently, it is essential to develop a simple, economical, and efficient method for the analysis of pharmacologically active

metabolites, like the affinity-directed fractionation-mass spectrometry methods used in the present investigation (Fan et al. 2019; Rosas-Ramírez et al. 2020). Mexico ranks first in the world in childhood obesity, second in adults, and sixth in prevalence of diabetes, one of the main causes of blindness, chronic kidney failure, and non-traumatic amputations (Turnbull et al. 2019), which complicates their medical care when facing an emerging disease, such as COVID-19 (Pal and Bhansali 2020). The prevalence of diabetes in the country is the cause of a national mortality alert.

In the present research, α -glucosidase from the yeast *Saccharomyces cerevisiae* and glucose-6-phosphatase (G6Pase) from liver microsomes of rats were selected as the enzymes; acarbose and chlorogenic acid as the high-affinity inhibitors for positive controls (Fig. S1); and a total ethanol/water root extract from the traditional-medicinal plant *Smilax moranensis* M. Martens & Galeotti, Smilacaceae, as the source of high-affinity inhibitors. These two key enzymes involved in the control of the homeostatic regulation of blood glucose levels with reduction of postprandial glycemic peaks (Ghosh et al. 2002). The hypoglycemic effect on rats of the ethanol/water extract has been previously reported (Andrade-Cetto 2011; Romo-Pérez et al. 2019; Mata-Torres et al. 2020) and 3-*O*-caffeoyl-quinic acid, 5-*O*-caffeoyl-quinic acid, and trans-resveratrol were isolated, which may potentially be involved in this effect (Romo-Pérez et al. 2019). *Smilax*

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moranensis is native to Mexico, known as *sarsaparilla*, corresponding to a complex of species of the *Smilax* genus used in traditional medicine since pre-Columbian times. Besides the use as hypoglycemic, it has expectorant, diuretic, depurative, and anti-inflammatory properties (Andrade-Cetto 2011; Romo-Pérez et al. 2019).

Materials and Methods

Low-resolution electrospray ionization mass spectrometry (ESI-MS) data were measured in a Bruker Daltonics Esquire 6000 ESI ion trap mass spectrometer. Nitrogen was used as both nebulizing and drying gas. Mass spectra were acquired over the range of 50–1500 m/z in 10 s/scan using electrospray ionization with a capillary voltage set to 4 kV. Direct infusion ESI-MS spectra were deconvoluted using the Bruker Data Analysis software. High-resolution ESI-MS data were measured in a coupled liquid chromatography system with single quadrupole mass spectrometry and time of flight (HPLC-EM-SQ-TOF Model G6530BA) (Agilent Technologies, Inc., Santa Clara, CA, USA).

The roots of *Smilax moranensis* M. Martens & Galeotti, Smilacaceae, were originally collected in Santos Reyes Nopala, Oaxaca, Mexico, in 2011, and a voucher specimen was deposited in the Medicinal Plant Herbarium IMSS (IMSS 15815); for the chemical study, new fresh plant material was collected in the same location on 2013 and its authenticity has been determined by pharmacopeial methodologies and comparison with the authentic samples on deposit at the Herbarium IMSS (Andrade-Cetto 2011). After collection, the powdered dry roots (0.1 kg) were macerated at 30 °C in a mixture (500 ml) of EtOH/H₂O (1:1) for 2 h to give, after removal of the solvents, a dark brown syrup (2 g).

The affinity-directed fractionation-mass spectrometry methods used in the present investigation were previously described (Rosas-Ramírez et al. 2020). Aliquots (10 µl, in triplicates) of the EtOH/H₂O-soluble extract (200 µg/ml), as well as of chlorogenic acid (positive control for G6Pase) and acarbose (positive control for α-glucosidases) at a concentration of 2000 µM, were independently incubated for 5 min with 20 µl of the enzyme stock solution (Andrade-Cetto and Cárdenas 2010; Rosas-Ramírez et al. 2018, 2020). Upon loading the test samples at the top of the spin-exclusion column, the mixtures were eluted by centrifugation at RCF 42,985×g for 4 min; then, the eluate (50 µl), corresponding to the solvent front and containing the enzyme-inhibitor complex, was collected and treated as previously described by denaturing acidic conditions (Rosas-Ramírez et al. 2020). They were analyzed by direct flow injection on an ion trap ESI mass spectrometer.

Analytical HPLC analyses were performed on an Agilent 1260 Infinity system equipped with a G1311B quaternary pump, a G1367E auto sampler, and a G1315C DAD. The

detector was maintained at 230, 254, 280, 320, and 365 nm. The separation was carried out using a Phenomenex® Luna Polar C-18 (50 × 2.1 mm id., 1.6 µm) reversed-phase column; the control of the equipment, data acquisition, and processing and management of the chromatographic information were performed by OpenLab CDS ChemStation Edition (2001–2013) software. The HPLC-DAD conditions for the EtOH/H₂O-soluble extract and the free ligand from the α-glucosidase-EtOH/H₂O extract complex were as follows: flow rate of 0.30 ml/min with water containing 0.1% formic acid as solvent A and CH₃CN as solvent B using a gradient elution of 94:06 (A:B) at 0–15 min, 88:12 (A:B) at 15–30 min, 78:22 (A:B) at 30–40 min, 70:30 at 40–48 min, 05:95 (A:B) at 48–49 min, and holding for a minute and again returning to the initial conditions 94:06 (A:B) at 35 min. The column temperature was kept at 35 °C. Working solutions for the EtOH/H₂O-soluble extract were prepared by dissolving 20 mg in 2 ml of a mixture of MeOH/H₂O (1:1). The samples were injected (3 µl) using an autosampler. Chlorogenic acid was identified by comparison using the HPLC retention time, analyzing their absorbance spectrum profile and co-elution experiments.

Docking was carried out with Auto Dock 4.2 software (The Scripps Research Institute, La Jolla, CA, USA) using the default parameters as previously described (Rosas-Ramírez et al. 2018).

Results and Discussion

Affinity-directed fractionation, through the use of size exclusion chromatography (SEC) on spin column centrifugation for the purification of proteins of therapeutic interest for screening of compound libraries of natural extracts, was applied in combination with ESI-MS for ligand detection (Siegel 2007; Rosas-Ramírez et al. 2020) to identify G6Pase and α-glucosidase inhibitors from *S. moranensis* roots. First, rat hepatic G6Pase was selected as the enzyme and chlorogenic acid as a high-affinity inhibitor (Fig. S2). Figure 1 illustrates the LRESI-MS spectra obtained from these SEC/ESI-MS affinity-screening assays: G6Pase, showing the enzyme peaks (Fig. 1a); and the free ligand adduct (m/z 380 [$M - O + C_2H_5N$][−]) from the G6Pase-chlorogenic acid complex after elution in SEC and released by denaturing acidic conditions, where the enhanced inhibitor peak was tracked by the applied SEC/ESI-MS affinity-directed fractionation (Fig. 1b). Thus, this procedure was applied to the EtOH/H₂O-soluble extract from *S. moranensis* roots; the LRESI-MS of the free ligand adduct from the G6Pase-EtOH/H₂O extract complex (Fig. 1c) shows the same peak obtained from the complex G6Pase-chlorogenic acid (m/z 380), which indicates that chlorogenic acid is the inhibitor of the G6Pase in *S. moranensis*, besides, it was the only metabolite observed in LRESI-MS after the affinity-directed fractionation. The 26-amu difference

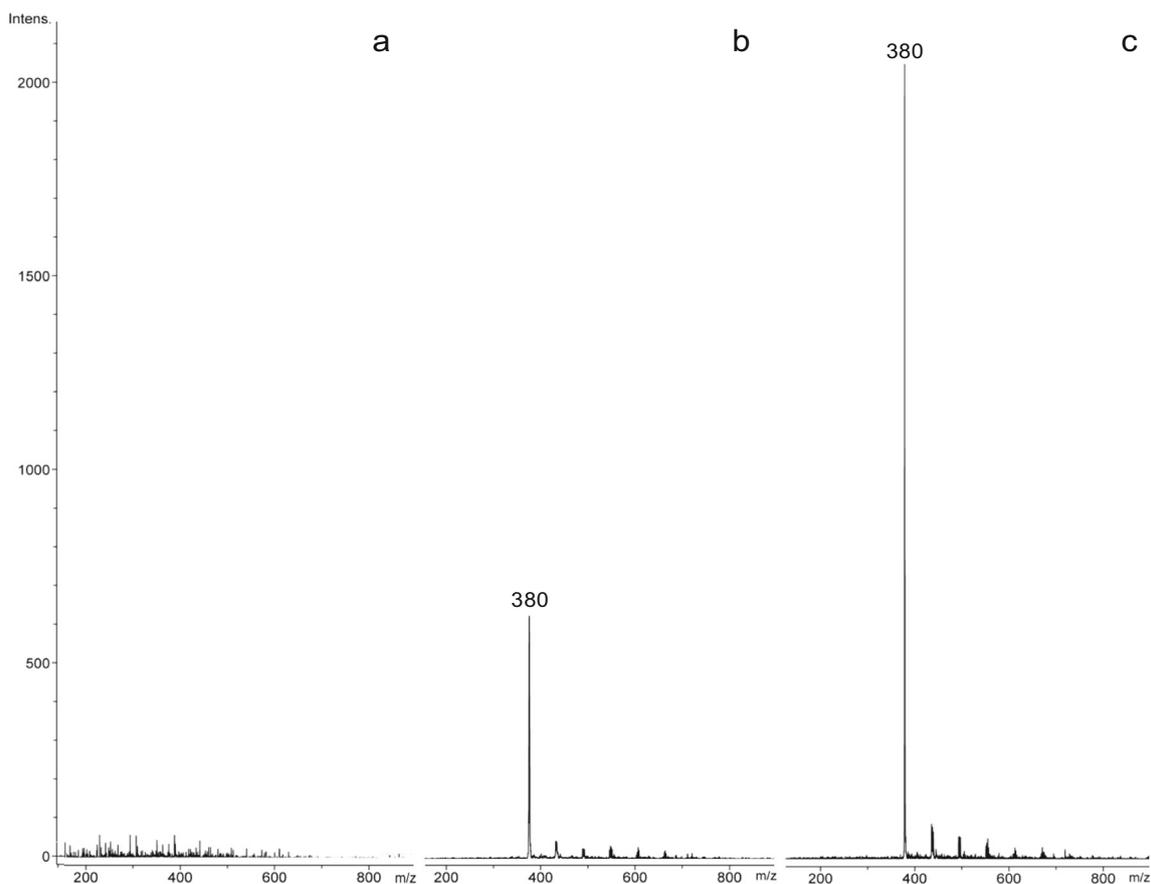


Fig. 1 Low-resolution ESI mass spectra, in negative mode, obtained from the affinity-screening assay for G6Pase from liver microsomes: (a) LRESI-MS of G6Pase, showing the enzyme peaks after affinity assay without inhibitor; (b) LRESI-MS of free ligand adduct (chlorogenic acid, $C_{16}H_{18}O_9$ plus amine group: m/z 380 $[M - O + C_2H_5N]^-$, $C_{18}H_{23}O_8N^-$

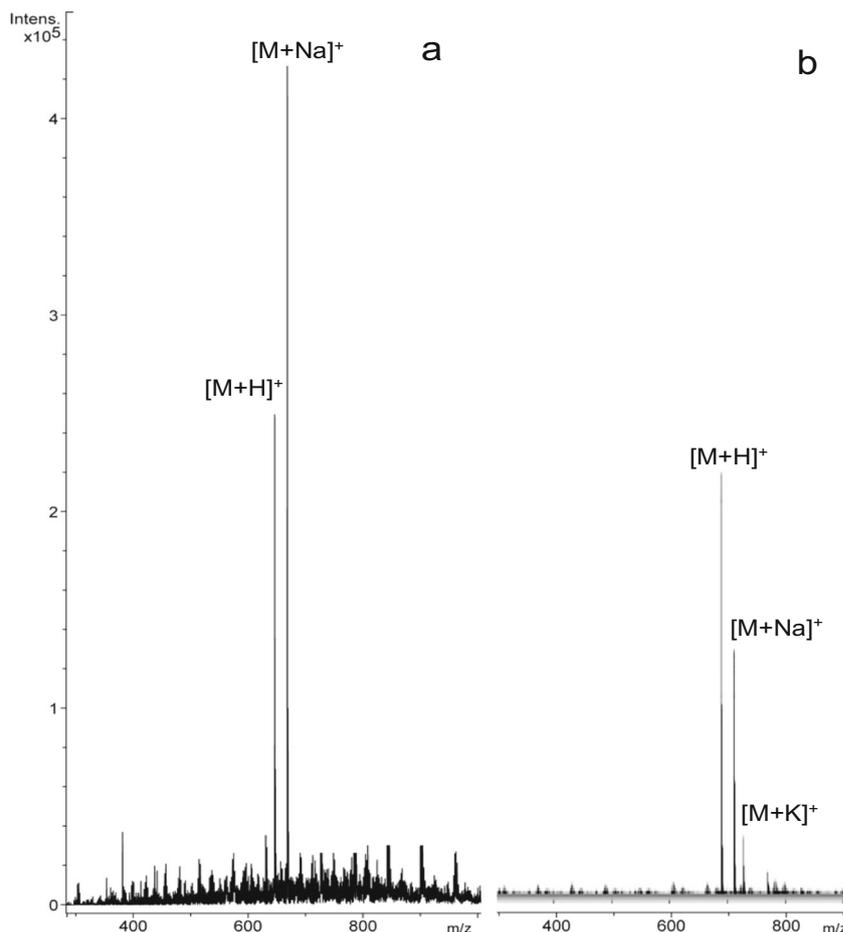
H) from the G6Pase-chlorogenic acid complex by the applied affinity-screening assay; (c) LRESI-MS of free ligand (m/z 379) from the G6Pase-ethanol/water extract complex, showing the inhibitor peak tracked by the applied affinity-screening assay

between the m/z peak of the chlorogenic acid alone (m/z 353 $[M - H]^-$; $C_{16}H_{18}O_9 - H$) and the m/z 380 peak of the G6Pase-chlorogenic acid complex (Fig. S2), after elution in SEC and released by denaturing acidic conditions, indicates the formation of a Schiff base adduct with an amine residue in lateral protein chains such as alanine (Fig. S3), as previously proposed for the irreversible inhibition of this enzyme (Arion et al. 1997). HPLC analysis was carried out with the EtOH/H₂O extract and the chromatographic profile indicated that the chlorogenic acid is the main metabolite present in the extract (Fig. S4a). Extensive investigations of the interactions of chlorogenic acid with the components of the rat hepatic G6Pase system determined that binding of chlorogenic acid is freely reversible with G6P-specific translocase T1 (Arion et al. 1997), which mediates diffusion of the G6P through the membrane. G6Pase catalyzes the terminal reaction of glycogenolysis and gluconeogenesis, the two glucose-producing pathways in the liver of humans and higher animals (Arion et al. 1997). G6Pase is widely held to be a significant factor in

the abnormally high rates of hepatic glucose production observed in the diabetic state (Mata-Torres et al. 2020).

For the affinity-screening assay of α -glucosidase from *S. cerevisiae*, Fig. 2 illustrates LRESI-MS obtained from these SEC/ESI-MS affinity-screening assays: LRESI-MS of the free ligand (acarbose $C_{25}H_{43}NO_{18}Na$: m/z 668 $[M + Na]^+$) from the α -glucosidase-acarbose complex (Fig. 2a), high-resolution ESI-MS obtained of the free ligand (m/z 689.2044 $[M + H]^+$; $\delta = -4.7$ ppm) from the α -glucosidase-ethanol/water extract complex showed the inhibitor peak (Fig. 2b). Other minor signals observed are related to $[M + Na]^+$ and $[M + K]^+$ ions of the inhibitor, m/z 711.4189 and 727.1639, respectively. Five isomeric flavonolignan glycosides with the same molecular weight of 688 g/mol ($C_{33}H_{36}O_{16}$) have previously been reported (Fig. S5): flavonolignans diastereoisomeric of triclin 4'-*O*-(erythro- β -guaiacylglyceryl) ether 7-*O*-glucoside and triclin 4'-*O*-(threo- β -guaiacylglyceryl) ether 7-*O*-glucosides, from leaves of *Hyparrhenia hirta* (Bouaziz et al. 2002); diastereoisomeric triclin 4'-*O*-(threo- β -guaiacylglyceryl) ether

Fig. 2 Low-resolution ESI mass spectra obtained from SEC/ESI-MS affinity-screening assay for intestinal acetone powder from rats and α -glucosidase from yeast. **(a)** LRESI-MS of free ligand (acarbose $C_{25}H_{43}NO_{18}Na$: m/z 668 $[M + Na]^+$) from the α -glucosidase-acarbose complex, by the applied affinity-screening assay intestinal acetone powder from rats. **(b)** HRESI-MS spectra of free ligand (m/z 689.2044 $[M + H]^+$) from the α -glucosidase-ethanol/water extract complex, by the applied affinity-screening assay with α -glucosidase from *Saccharomyces cerevisiae*

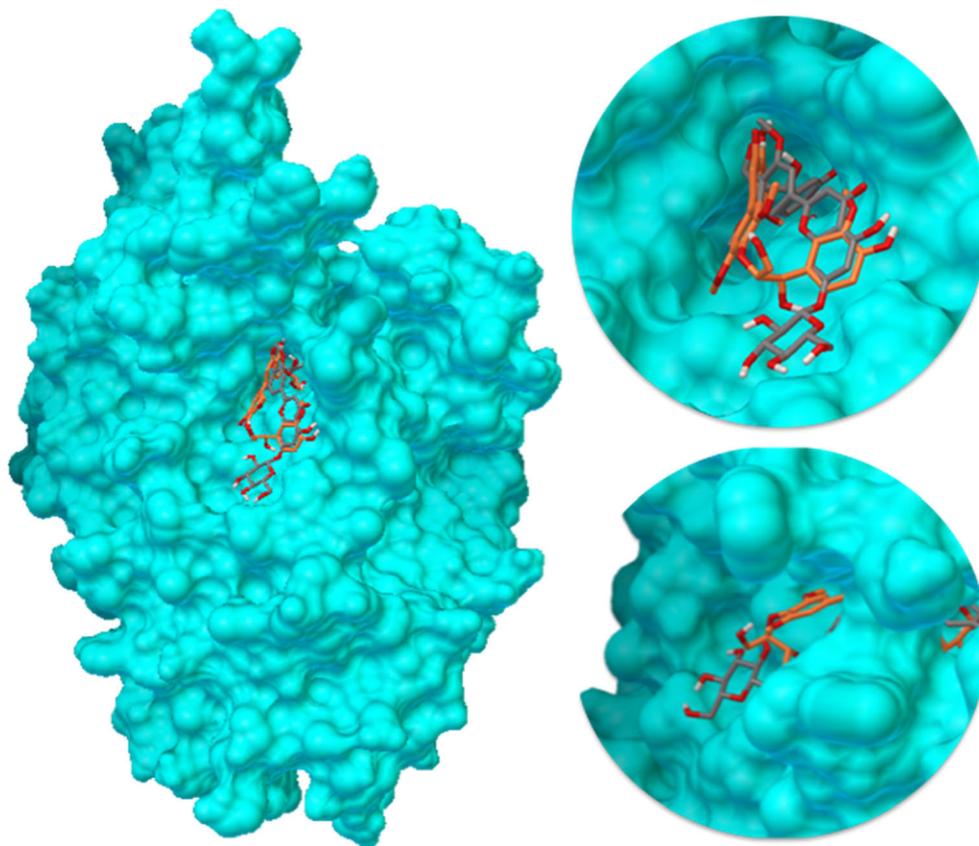


together with their 7''-*O*-glucosides, from leaves of *Zizania latifolia* (Lee et al. 2015); and the isomer tricrin 4'-*O*-(erythro- β -guaiacylglyceryl) ether 9''-*O*- β -D-glucopyranoside from the aerial parts of *Oryza sativa* (Yang et al. 2014). Affinity-screening assays coupled off-line to HPLC (Fig. S4b) were used to analyze their absorbance spectrum profiles of the high-affinity compound (R_t 38.70 min, water containing 0.1% formic acid/ CH_3CN (94:06) UV λ_{max} : nm: (242, 278, and 326), revealing that they correspond to this class of flavonolignans (Bouaziz et al. 2002; Yang et al. 2014; Lee et al. 2015). However, more studies are required to verify the structure. The flavonolignans observed were analyzed by docking studies and a high affinity for the active site was observed, finding greater interaction of tricrin 4'(erythro and threo)-7-*O*-glucoside with the α -glucosidase enzyme.

Docking studies were carried out to improve our understanding of the interaction of active compounds inside MAL12, which was selected as the template for molecular modeling to establish a comparison between the results from the affinity-screening assay and the theoretical inhibition constant (K_i) obtained from *in silico* studies. Ligand-receptor molecular models were calculated for chlorogenic acid and

acarbose, previously reported inhibitors of α -glucosidase (Arion et al. 1997; Playford et al. 2013), to test the adopted protocol with the α -glucosidase template (Fig. S6). The first analysis predicted that acarbose and tricrin 4'(erythro and threo)-7-*O*-glucoside fit well in the catalytic pocket in the hypothesized binding model and showed hydrogen-bonding interactions with the amino acid residues His279 (1.94 Å) and Gln322 (1.71 and 2.19 Å) for acarbose (Fig. S6), Ser156 (2.71 Å) and Gln322 (2.01 Å) for tricrin 4'-erythro 7-*O*-glucoside, and His279 (1.92 Å) and Ser308 (1.82 Å) for tricrin 4'-threo-7-*O*-glucoside. Chlorogenic acid formed hydrogen bonds in a different place from the active site in MAL12 (Fig. S6) with Lys155 (2.14 Å), Asp429 (2.08 Å), and Phe430 (1.85 Å). Optimized docking results using MAL12 with tricrin 4'(erythro and threo)-7-*O*-glucoside showed hydrogen-bonding interactions with the amino acid residues Phe157 (2.23 Å), His279 (1.89 Å), Thr301 (2.07 Å), and Arg312 (2.02 Å) for tricrin 4'-erythro-7-*O*-glucoside, and His279 (1.91 and 2.11 Å), Asp282 (2.00 Å), Glu304 (1.98 Å), and Ser329 (1.71 Å) for tricrin 4'-threo-7-*O*-glucoside (Fig. 3). The theoretical inhibition constant (K_i) was 33.29 μ M for tricrin 4'-erythro and 39.93 μ M for tricrin 4'-

Fig. 3 Docking results using the structural model of α -D-glucosidase (MAL12) from *Saccharomyces cerevisiae*, which comprises the catalytic site of the enzyme and the binding interactions for triclin 4'-erythro-7-O-glucoside (gray) with the residues Phe157, His279, Trh301, and Arg312, and for triclin 4'-threo-7-O-glucoside (orange) with the residues His279, Asp282, Glu304, and Ser308



threo. Based on the theoretical docking results for chlorogenic acid, the binding site was situated in a place away from the active site of the template selected for molecular modeling, and projection corresponded to the non-competitive inhibition mode (Pérez-Nájera et al. 2018). The theoretical inhibition constant (K_i) for the chlorogenic acid showed the largest inhibition constant of 135.73 mM for MAL12 in comparison with acarbose (51.4 nM) and the diastereoisomers of triclin 4'-*O*-(erythro/threo- β -guaiacylglyceryl) ether 7-*O*-glucoside.

The identification of flavonolignans contributed to expand the knowledge of the active α -glucosidase inhibitors present in the decoctions of *sarsaparilla* (*Smilax moranensis*) used to treat hyperglycemia, since previous studies only identified as the major therapeutical marker the well-known chlorogenic acid (Andrade-Cetto 2011; Romo-Pérez et al. 2019). Note that the pharmacological potential of the analyzed plant material is probably due to the capability of polyphenols to interact with important cellular processes in which key enzymes such as cyclooxygenase, lipooxygenase, phospholipase A₂, NADH-oxidase, or glutathione reductase are involved. Catechols in polyphenols are also able to form chelates with metals and are reactive in their oxidized form (like quinonas) with nucleophiles present in lateral protein chains such as cysteine and lysine. Therefore, further *in vivo* studies with the dereplicated flavonolignans are needed to understand if they can be used as novel α -glucosidase inhibitors.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s43450-020-00116-3>.

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Authors' Contribution DRR designed the study and performed the affinity-directed fractionation studies, SEC-EM experiments, *in vitro* inhibitory enzymatic assays, and molecular docking, as well as elaborated the first draft of the manuscript. SER performed the HPLC analysis and supervised the bioassays and molecular docking. AAC designed and supervised bioassays, supervised HPLC methods, and provided laboratory instrumentation and supplies. RAE designed and supervised bioassays, and provided laboratory instrumentation and supplies. All of the authors have read the final manuscript and approved the submission.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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