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Research Paper

Effect of a Standardized Hydroalcoholic Extract of *Echeveria subrigida* on Mice Glycemia and *In Vitro* Kinetics of α-Glucosidase Inhibition

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Leaf extracts of *Echeveria subrigida* show antioxidant, antibacterial, anti-mutagenic, and α-glucosidase inhibitory activities. The inhibitory α-glucosidase is associated with the content of quercetin-3-O-glucoside and isorhametin-3-O-glucoside. The aims of this study were to analyze the effect on mice glycemia and *in vitro* kinetics of α-glucosidase inhibition of the hydroalcoholic extract of *Echeveria subrigida* standardized in the content of isorhametin-3-O-glucoside. The high-performance liquid chromatography method was validated to quantitate isorhametin-3-O-glucoside. Hydroalcoholic extract of *Echeveria subrigida* identity was characterized by its organoleptic, physicochemical, pharmacological and toxicological parameters. The effects on glycemia were carried out in 6 w old male Balb-C mice and using the following treatments; hydroalcoholic extract of *Echeveria subrigida* at 50, 100 and 200 mg/kg b.w.; positive control glibenclamide or acarbose at 10 mg/kg b.w. The inhibition kinetics of hydroalcoholic extract of *Echeveria subrigida* was determined by the Lineweaver-Burk plots. Hydroalcoholic extract of *Echeveria subrigida* had 4.87±0.14 mg isorhametin-3-O-glucoside/g, complied with the World Health Organization parameters for standardized extracts and induced a mixed inhibition on α-glucosidase. Hydroalcoholic extract of *Echeveria subrigida* (200 mg/kg b.w) and glibenclamide treatments showed similar percent hypoglycemia, 49.1 % and 52 %, respectively. The anti-hyperglycemic assay showed that similar percent reductions in glucose-levels were found in the treatments with hydroalcoholic extract of *Echeveria subrigida* (100 mg/kg, 29.32 %; 200 mg/kg, 28.99 %) and acarbose (10 mg/kg, 19.87 %). The standardized hydroalcoholic extract of *Echeveria subrigida* showed hypoglycemic and anti-hyperglycemic activities, stability, and innocuity, suggesting its potential to prevent/treat diabetes mellitus.

Key words: Antidiabetic activity, ethanolic extract, standardized extract, *Echeveria subrigida*, high-performance liquid chromatography method validation

Diabetes Mellitus (DM) is characterized by the loss of glucose homeostasis and alteration of insulin signaling and metabolism of carbohydrates and lipids, leading to progressive systemic disorders (hyperlipidemia, hyperglycemia, nephropathy, hepatic damage, vascular dysfunction). DM affects more than 425 million people worldwide, and about 90 % of them have type 2 DM (DM2), which is associated with obesity and a sedentary lifestyle[1]. Different drugs are used to treat DM2 (insulin secretagogues, insulins, inhibitors of glucosidases)[2], but many diabetic patients use traditional/complementary medicine[3].

Different plant natural products contain compounds with hypoglycemic properties (flavonoids, alkaloids, steroids, peptides) and potential to be developed as new anti-diabetic drugs. Flavonoids (flavones and flavonols) are potent inhibitors of α-glucosidase, showing different inhibition kinetics[4]. Plant extracts (*Cinnamomum zeylanicum*, *Stevia rebaudiana*) have anti-diabetic properties,

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showing inhibitory activity of α-glucosidase and suppression of the hyperglycemic response in diabetic rats[5]. In fact, several plant standardized extracts have been evaluated to treat DM (*Cleome droserifolia*, *Quassia amara* L., *Withania somnifera*)[6,7]. A standardized extract contains constant levels of active ingredients, induces predictable pharmacologic and physiologic effects and shows identity characteristics and therapeutic efficacy. Thus, the method of quantification used in the standardization process must be validated and the physical, chemical and pharmacological properties of the standardized extract must be established[8].

Echeveria subrigida (*E. subrigida*) (BL Rob. & Seaton) Rose (Crassulaceae) is a plant native to Mexico. Its leaves contain active metabolites, nutritional components (ascorbic acid, tocopherol, β-carotene), organic acids (malic, malonic, succinic), fatty acids (linoleic, palmitic), carbohydrates (fructose, altrose, sucrose), terpenes (α-amirine, campesterol, germanicol, sitosterol), and phenolics (flavonoids, tannins), among others[9-11]. Leaf extracts of *E. subrigida* show relevant activities for the human being like antibacterial against human pathogens, antioxidant, antimutagenic and Inhibitory of α-Glucosidase (IαG). The methanol extract of *E. subrigida* leaves has higher IαG activity than acarbose[10], and the flavonoids isorhamnetin-3-O-glucoside (I3G), quercetin-3-O-glucoside (Q3G), and proanthocyanidins are the main compounds responsible for the IαG activity[12]. These studies support the potential of *E. subrigida* extracts to treat DM2, but the employed extracts were obtained with methanol and not standardized, besides, the *in vivo* effect on glycemia and the inhibitory mechanism of α-glucosidase have not been established. Consequently, the aims of this study were to standardize a Hydroalcoholic Extract of *E. subrigida* (HE-Es) in its content of I3G, to evaluate the extract quality properties (pharmacognosy, physicochemical, and toxicological), to establish its effect on the basal and postprandial levels of glucose on normoglycemic Balb-C mice, and to determine its *in vitro* inhibition kinetics on α-glucosidase.

MATERIALS AND METHODS

Plant material:

Leaves of *E. subrigida* Rose were collected in October 2018 near the town of El Palmito, Concordia, Sinaloa, Mexico 2000 m a.s.l.; 23°34′06″ N, 105°50′53″O). Dr. Rito Vega Aviña authenticated the plant material and deposited one specimen (11742) in the herbarium of the School of Agronomy of the Autonomous University of Sinaloa. The leaves were washed (93.8±0.2 % moisture), cut, freeze-dried (VirTis 25EL, VirTis Co. U.S.), and milled to obtain a fine powder (mesh 40). The powder was stored at 20° in darkness until its use.

Animals:

Balb-C mice were obtained from Bioinvert company (Bioinvert, SA of CV, Mexico) and maintained at 24±2°, 50 % relative humidity, and light/dark cycles of 12 h. Water and feed (Nutricubos, Purina S.A. of C.V., Mexico) were provided *ad libitum*. The animal management was carried out according to the Official Mexican Standard NOM-062-ZOO-1999 and permission was obtained from the Institutional Ethical Committee constituted for the purpose.

Reagents and solvents:

Solvents were High-Performance Liquid Chromatography (HPLC) grade (TEDIA, U.S.). Bacteriological media were from Becton Dickinson: agars, soy trypticase, Baird-Paker, salt and mannitol, MacConkey, citrate deoxycholate, red violet bile, Sabouraund; and broths, soy trypticase, tetrathionate brilliant green bile, peptone water, lactose and Mossel. The following reagents were from Sigma Aldrich (St. Louis, MO, U.S.): quinine chlorohydrate, ethylenediaminetetraacetic acid, ferric ammonium sulfate, catechin, Triton X-100, chlorohydric acid, α-glucosidase from *Saccharomyces cerevisiae* (EC number 3.2.1.20), p-Nitrophenyl Glucopyranoside (p-NPG), p-Nitrophenol (p-NP), acarbose and glibenclamide.

Preparation of the HE-Es:

HE-Es was prepared by maceration in 80 % ethanol of the *E. subrigida* powder (1:10 w/v) with continuous stirring (150 rpm/3 d). The solvent was changed daily and the hydroalcoholic phases were mixed. Ethanol was eliminated under reduced pressure at 40° (BÜCHI Labortechnick AG, Switzerland), vacuum oven heating at 40° (Prendo SS-250, Mexico), and freeze-drying. The residue (HE-Es) was stored at -20°, under N_2 atmosphere and in darkness until use.

Standardization and quality control of the material/ extract:

The quantitative analysis of the active principles in HE-Es and the determination of the identity parameters (i.e., organoleptic, physicochemical, pharmacologic, and toxicologic) of the *E. subrigida* powder and HE-Es were carried out as described in the quality control methods for medicinal plant materials of the World Health Organization[13].

Standardization of the HPLC method:

HPLC conditions: The HPLC analyses were performed using an HPLC-DAD 1100 system (Agilent Technologies, U.S.) provided with an ACE EXCEL C18-Amide column (150×30 mm×3 µm) (Advanced Chromatography Technologies, U.K.). The mobile phase consisted of 1 % formic acid (A) and acetonitrile (B). 0.5 % B, linear gradient to 30 % B in 10 min, isocratic 10 min, linear gradient to 60 % B in 10 min, and isocratic 5 min. The separation conditions were as follows; running time 35 min, flow 0.4 ml/min, injection volume 15 µl, and detection at 355 nm[10].

Validation of the analytical method:

The parameters of the HPLC method (suitability, specificity, accuracy, precision and linearity) were validated as recommended by the United State Food and Drug Administration (FDA)[14]. The marker compound was I3G, and some parameters were also evaluated for Q3G.

Preparation of the sample and standard:

HE-Es was dissolved in MeOH (40 mg/ml). A C18 cartridge (CHROMAFIX, U.S.) was conditioned with 2 ml 50 % MeOH and 500 µl of the HE-Es were passed through the cartridge. Phenolics were eluted with 4 ml of MeOH into a 5 ml volumetric flask, and the volume was made up with MeOH. The solution was passed through a PVDF filter (17 mm, 0.45 µm, TITAN, U.S.) before HPLC analysis. Stock solutions of the standards (i.e., I3G, Q3G, and quercetin) were prepared in MeOH (1 mg/ml) and stored in amber vials at 4°. For analysis, dilutions in MeOH (1-100 µg/ml) were prepared from the stock solutions.

System suitability:

The system suitability was established by repeated injections (n=6) of 15 µg/ml I3G. The following parameters were calculated, Relative Standard Deviation (% RSD) for the retention time and peak area, the capacity factor (k'), the theoretical plate number (N) and the asymmetry factor (T). Peak resolution was determined for the HE-Es.

Specificity:

Solutions of the blank, standard (I3G), and HE-Es were analyzed by the established HPLC method. The method is specific if there is no signal in the blank or sample which interferes with the marker compound; besides, the Resolution Factor (Rs) (>1.5) and the peak purity are important parameters for the method specificity.

Linearity:

Linearity was measured by three injections of five different concentrations (6.25-100 µg/ml) of the marker compounds I3G and Q3G (1-25 µg/ml). Calibration curves were obtained for these compounds (R^2>0.99) and used to calculate the Limits Of Detection (LOD) and Limits Of Quantitation (LOQ).

Accuracy:

The accuracy was calculated from the Recovery Percentage (% R) of a compound similar to the analyte. The sample (40 mg/ml) was mixed with different concentrations of the Internal Standard (IS) quercetin (80, 100, and 120 % of the analyte concentration in the sample). The mixtures were processed as established in the section "Preparation of the sample and standard", and three independent injections per IS concentration were analyzed. The quantity of recovered IS was calculated using the IS calibration-curve (5-40 µg/ml). Results were reported as the average of % R for the three concentrations. The average recovering is acceptable if it ranges from 98 % to 102 %.

Precision:

The intraday precision (repeatability) was calculated from nine measurements (three concentrations and three repetitions each) of I3G and Q3G; they were carried out the same day using the previously described HPLC conditions. The interday precision was calculated using new solutions prepared at the same concentrations, which were analyzed on different days by a different analyst. The method's precision was reported as the average RSD, RSD ≤2 % is considered acceptable.

Quantitation of the active compounds by HPLC:

Flavonoids I3G and Q3G were purified and characterized as previously described[12], and calibration curves were prepared. Flavonoids were quantitated by HPLC as described in the previous section. Results were reported as mg of I3G and Q3G per g of HE-Es (mg/g of HE-Es).

Evaluation of quality parameters:

Organoleptic and physicochemical parameters: The organoleptic (color, odor, odor type, texture and particle size) and physicochemical (total ash, acid-insoluble ash, water-soluble ash and moisture) parameters of *E. subrigida* powder and HE-Es were determined as identity characteristics[13]. Color was also determined using a colorimeter (Chroma Meter CR-200, Minolta, Japan).

Pharmacological parameters: The bitterness of the HE-Es was measured by comparison with diluted solutions of quinine chlorohydrate. The assay was carried for only one person. Stock solutions of the quinine chlorohydrate standard (10 µg/ml) and sample (1 mg/ml) were prepared in drinking water (20-25°), as well as the nine dilutions indicated in the method[13]. The measurement was carried out by alternating the tasting of different quinine chlorohydrate solutions (4.2-5.8 µg/ml) and HE-Es starting with dilution five to continue with lower or higher dilutions depending on the bitterness. The standard concentration corresponding with the HE-Es bitterness was established as follows, lateral and superior parts of the tongue were exposed to 10 ml of each concentration solution for 30 s, the solution was spat out and the mouth was rinsed with drinking water between tastings. The bitterness was reported as units of quinine; one unit corresponded to a solution containing 1 in 2000 parts of quinine chlorohydrate and was calculated using the equation.

Bitterness in units/g=(2000×C)/(A×B),

where A=Concentration of the extract dilution (mg/ml), B=Volume (ml) per 10 ml used to prepare the dilution of the sample's minimal bitterness concentration, and C=Quantity of the quinine chlorohydrate contained in the dilution of the initial test's minimal bitterness concentration.

Hemolytic properties: Hemolytic activity was determined as described by Dima *et al.*[15] with some modifications. Blood samples of two healthy individuals were obtained in tubes added with ethylenediaminetetraacetic acid. After centrifugation (200 g/5 min) (EBA 20 Hettich Zentrifugen, Germany), the pellet was recovered, washed and centrifuged three times with Phosphate Buffered Saline ((PBS) 0.1 M, pH 7.4), resuspended in PBS, and incubated at 37° for 5 min. Then, 500 µl of the suspended cells were mixed with 500 µl of HE-Es (1 mg/ml), incubated at 37° for 30 min, and centrifuged at 200 g for 10 min. The free hemoglobin in the supernatant was measured at 540 nm in a microplate reader (Multiskan Bichromatic, Fisher Scientific, U.S.). Samples were analyzed by triplicate. The controls were Triton X-100 (1 % v/v) (positive) and PBS (negative).

The hemolysis percentage was calculated using the equation;

Percentage hemolysis=(Abs of sample/Abs of the positive control)×100

Bitterness properties: Condensed tannins were quantitated as described in the laboratory manual "Quantification of Tannins in Tree Foliage"[16]. The reaction mix consisted of 0.125 ml of HE-Es (20 mg/ml), 0.750 ml of acidified butanol (butanol:HCl 95:5 v/v), and 25 µl of ferric reagent (2 % of sulfate ammonium ferric in 2 N HCl, 1:1 v/v). The mixture was heated at 98±2° for 60 min in a heating block (Fisher Scientific, IN, U.S.) and then cooled to room temperature; a blank without sample maintained at room temperature was included for colour correction. The mixtures (100 µl) were transferred to a 96 well microplate and the absorbance was measured at 540 nm in a microplate reader. A calibration curve of catechin (0-5 mg/ml in MeOH) was used for quantification, and the results were reported as Catechin Equivalents (CE) (mg CE/g HE-Es).

Swelling index: The swelling index is the volume increase (ml) obtained after swelling 1 g of plant material under specific conditions. HE-Es (1 g), 1 ml of ethanol, and 25 ml of water were added into a 50 ml graduated cylinder; the initial volume was registered. The components were mixed with a stirring rod (10 s) every 10 min for 1 h and left to stand for 3 h. The final volume was registered, including any mucilaginous component if present. The assay was carried out by triplicate, and the average of the difference in volume was calculated.

Foam index: HE-Es (1 g) was mixed with 100 ml of

water. The mixture was boiled for 30 min, cooled, filtered and the volume was made up to 100 ml. Dilutions were prepared in graduated cylinders (1 to 10) in a final volume of 10 ml, the components were mixed vigorously for 15 s and left to stand for 15 min. The height (cm) of foam formed was measured, and the foam index was calculated as follows; foam index=1000/a; where a is the volume (ml) of the decoction used to prepare the dilution in the cylinder where the height was 1 cm.

Toxicological parameters:

Microbial contamination: The microbial contamination was determined as described in the "Quality Control Methods for Medicinal Plant Materials"[13]. The total count of bacteria and fungi and the presence of pathogens as enterobacteria and other Gram (-) (*Escherichia coli*, *Salmonella* spp., *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) were determined.

Aflatoxins: Aflatoxins were measured by High Performance Thin Layer Chromatography (HPTLC, CAMAG, Muttenz, Switzerland) as recommended in the application note A-12.4 of the CAMAG Company (Switzerland). The HE-Es (1 g) was dissolved in 75 % MeOH and filtered (Nylon 25 mm, 0.45 µm, Millipore, U.S.). The filtrate was fractionated by washing three times with 10 % NaCl and hexane. The aqueous phase was recovered, and the aflatoxins were obtained by washing twice with dichloromethane; the organic phase was recovered and concentrated using a rotary evaporator (BÜCHI R-124 Labortechnick, Germany). The sample was resuspended in 5 ml MeOH-water (60:40 v/v) and diluted with 5 ml of water. The solution was passed through an immunoaffinity column Easi-Extract Aflatoxin (10 cm×1 cm) (R-Biopharm Ag, Germany). The column was washed twice with PBS and the aflatoxins were eluted with 2 ml MeOH. The solvent was evaporated with N_2(g) and the residue was suspended in 200 µl of acetonitrile. A calibration curve was prepared with aflatoxin standards: B1, G1, B2, and G2 (Sigma-Aldrich, U.S.). The sample (10 µl) and standard (2, 5, 7.5, and 10 µl) were automatically applied (Linomat 5 HPTLC, CAMAG, Muttenz, Switzerland) onto a silica gel 60 F_{254} plate (20×10 cm) (Merck, Germany). The plate was developed with chloroform-acetone-water (140:20:0.3 v/v/v) in a chamber previously saturated with $MgCl_2$ for 20 min. After plate development and solvent evaporation, the chromatogram was obtained at 366 nm with the software visionCATS2.5.

Inhibitory activity of α-glucosidase and inhibition kinetics:

Determination of the half inhibitory concentration: The inhibitory activity of α-glucosidase was measured as previously reported[10]. The half Inhibitory Concentration (IC_{50}) was determined by mixing 100 µl of α-glucosidase solution (0.5 U/ml) in PBS (0.1 M, pH 6.9) with 50 µl of HE-Es at different concentrations to reach a final concentration of 20, 40, 60, 80, and 100 µg/ml. The microplate was incubated at 37° for 10 min (Stat Fax-2200, AWARENESS TECHNOLOGY, U.S.), added with 50 µl p-nitrophenyl-α-D-glucopyranoside (5 mM in PBS), and incubated again (37°/10 min). The absorbance was measured at 405 nm (Labsystems Multiskan RC, Fisher Scientific, Finland). Acarbose (0.5-4 mg/ml) was the positive control and the solution without sample was the reaction control. The percentage of α-glucosidase inhibition was calculated using the equation.

$$\text{Percentage inhibition}=[(Abs_C-Abs_M)/Abs_C]\times 100$$

where Abs_C is the absorbance for the control reaction, and Abs_M is the absorbance of the sample. The IC_{50} value was calculated with the inhibition percentages.

Determination of the enzyme-inhibition kinetics: The kinetics of α-glucosidase inhibition was determined with the Lineweaver-Burk method[17]. The enzyme activity was measured using different concentrations of p-NPG (0.625, 1.25, 2.5, and 5 mM) and HE-Es (0, $1\times IC_{50}$, and $2\times IC_{50}$). The reaction conditions are indicated in the previous paragraph, but the absorbance measurements were taken every minute for 5 min. The reaction was monitored by the p-NP formation, using a calibration curve (0.1875-6 mM). A double reciprocal curve (1/activity *vs.* 1/substrate concentration) was graphed to obtain the constants of Michaelis-Menten (K_m) and maximal Velocity (V_{max}).

Hypoglycemic activity of HE-Es on normoglycemic mice: The hypoglycemic activity was determined as reported[18] with some modifications. Five groups (n=6) of Balb-C mice were formed. group 1, negative control (saline solution); group II, positive control (glibenclamide 10 mg/kg b.w.); groups III, IV and V, HE-Es (50, 100,

and 200 mg/kg b.w., respectively). The basal glucose level was measured after 8 h fasting; immediately, treatments were administered orally and glucose levels were measured every h for 5 h. Blood was obtained by tail puncture, and glucose was measured with a glucometer (Accu-Chek® Roche, Mexico). Glucose levels were reported as mg/dl of blood.

Antihyperglycemic activity of the HE-Es on normoglycemic mice (oral glucose tolerance test): Oral glucose tolerance test was carried out as previously described[5] with some modifications. Five groups (n=6) of Balb-C mice were formed. Group I, negative control (saline solution); group II, positive control (acarbose 10 mg/kg b.w.); groups III, IV, and V, HE-Es (50, 100, and 200 mg/kg b.w., respectively). The basal glucose level was determined after 8 h fasting, and treatments were administered orally immediately. A half-hour later, mice received sucrose (2 g/kg b.w.), and the glucose levels were measured after 30, 60, 90, and 120 min. Blood was obtained by tail puncture, and glucose was measured with a glucometer (Accu-Chek® Roche, Mexico). Glucose levels were expressed in mg/ml of blood and as the incremental area under the blood glucose response curve (iABC) (mg.min/dl).

Statistical analysis:

The results were reported as the mean±Standard Error (SE) or Standard Deviation (SD). The hypoglycemic and antihyperglycemic activities were analyzed by one-way analysis of variance to establish the differences among and within treatments. Means were contrasted by the Dunnett test with a significance level of $p \leq 0.05$. The incremental area under the blood glucose response curve was calculated with the trapezoidal rule. Data was analyzed with the software Graphpad Prism 8.1.

RESULTS AND DISCUSSION

The extraction yield for the HE-Es was 21.80±0.85 % d.w. The HPLC method was suitable to quantitate the marker compound I3G in the HE-Es and complied with the FDA criteria[14] (Table 1). The method was specific since the other peaks did not interfere with that of I3G (fig. 1A-fig. 1C); the resolutions (R) with peaks adjacent to that of I3G were 1.6 and 2.3 (Table 1). Besides, the peak purity of I3G was above the limit (>990) (fig. 1D and fig. 1E). The HPLC method showed good accuracy, repeatability (% RSD<2 %), linearity (R^2>0.99), and its LOQ (I3G=0.25 µg/ml and Q3G=0.21 µg/ml) was lower than the content of marker compounds in the HE-Es solution (I3G=19.3 µg/ml, Q3G=12.04 µg/ml) (Table 2). According to the described characteristics, the developed HPLC method is adequate to standardize the HE-Es[14]. The main monomeric flavonoids with α-glucosidase inhibitory activity in HE-Es are I3G and Q3G[12]. The validated HPLC analysis of the HE-Es showed that 1 g of HE-Es contained 4.82±0.15 mg of the marker compound I3G and 3.01±0.03 mg of Q3G; these values were similar to those reported in a methanol extract of *E. subrigida*[9]. In this regard, different plant extracts have been associated with biological activities and standardized in the content of flavonoids. For example the methanol extract of *Balanites aegyptiaca*, standardized in the content of rutin (1.3 mg/g) and isorhamnetin (0.04 mg/g), shows antidiabetic activity, reducing the glucose absorption by C2C12 muscle cells[7]; on the other hand, the ethanol extract of *Carthamus tinctorius*, standardized in kaempferol-3-O-rutinoside (56 mg/g) and anhydrosafflor yellow B (57 mg/g), has neuroprotective activity in mice with Parkinson[19].

TABLE 1: SYSTEM SUITABILITY

Parameter		I3G (Mean)	HE-Es	Acceptance requirements
Retention time (RT)	Mean	20.238	-	
	% RSD	0.19		≤2 %
Peak area	Mean	4007.91	-	
	% RSD	1.93		≤2 %
Retention factor (k′)	5.55	-	>1	165±74.73ns
Column plate number (N)	87,097	-	>3000	165±74.73ns
Tailing factor (T)	1.09		<2	165±74.73ns
Resolution (Rs)1	-	1.6, 2.3	>1.5	165±74.73ns

Note: 1: Resolution calculated with respect to the peaks adjacent to the marker compound (isorhamnetin-3-O-glucoside, I3G) in the hydroalcoholic extract of *Echeveria subrigida* (HE-Es)

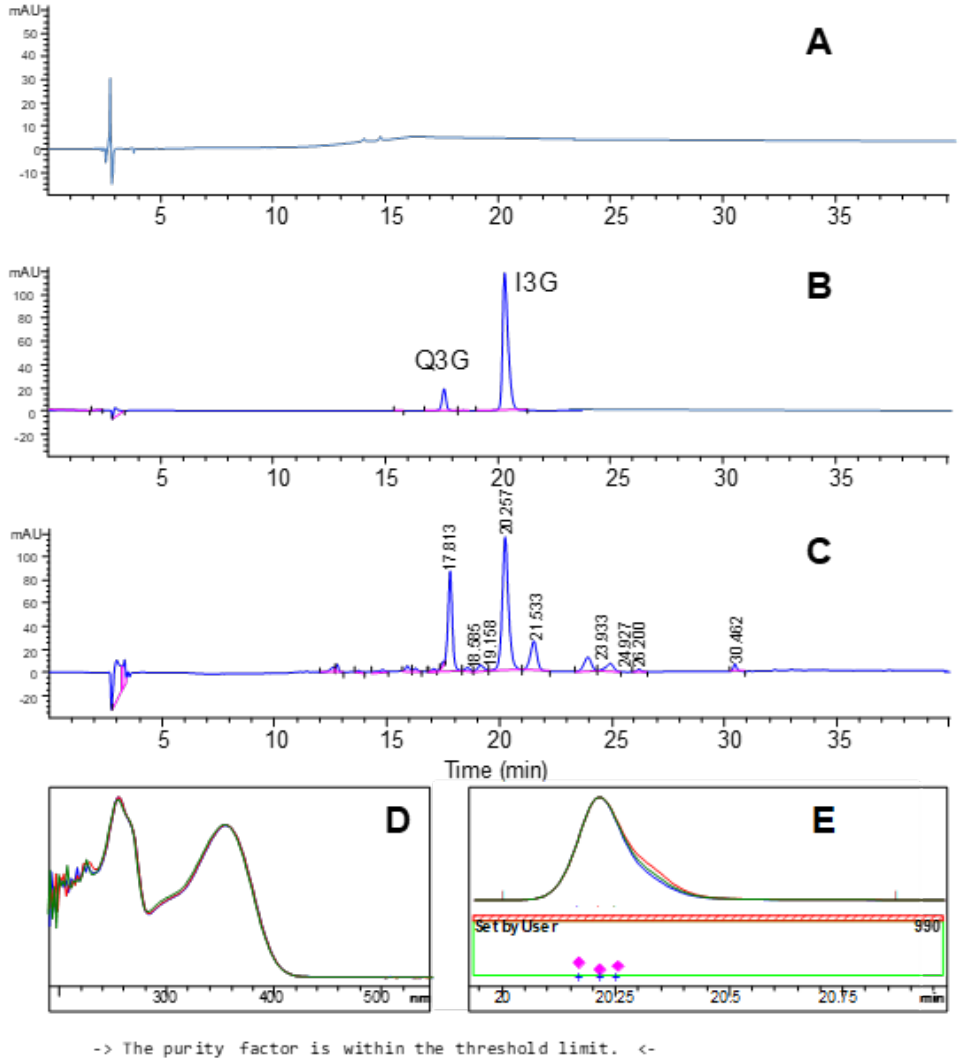

Fig. 1: HPLC chromatogram of (A): Blank, (B): Standards, and (C): extract solutions; (D): UV spectrum and (E): Peak purity of isorhamnetin-3-O-glucoside (I3G) in the extract

TABLE 2: METHOD VALIDATION PARAMETERS1

Parameters	I3G	Q3G
	Accuracy	
% Recovery2 (Mean)	95.3±0.91	
	Precision (% RSD)	
Repeatability (n=6)	1.59	1.73
Intermediate precision	1.43	1.57
	Linearity	
Range (µg/ml)	6.25–100	1.05–25
Regression equation	y=102.17x+219.6	y=100.97x+30.95
Coefficient of determination (R2)	0.9976	0.999
Limit of detection (µg/ml)	0.083	0.068
Limit of quantification (µg/ml)	0.25	0.21

Note: 1: I3G is isorhamnetin-3-O-glucoside and Q3G is quercetin-3-O-glucoside and 2Recovery percentage of quercetin internal standard, the mean of the three added concentrations is presented

The organoleptic characteristics of the *E. subrigida* powder and HE-Es showed clear differences in color, particle texture, and odor (Table 3). The aromatic-fruity odor of the HE-Es could be associated with esters, aldehydes, and ketones identified in *E. subrigida*[11]; these compounds were previously shown to provide a fruity odor[20]. The physicochemical analysis showed higher ash content in *E. subrigida* powder than in the HE-Es, mostly water-soluble in both materials, and hygroscopic characteristics for the HE-Es. The higher ash values in the flour can be associated with the loss of inorganic material during the extraction with ethanol (Table 3). In general, the ash represents the content of inorganic salts (silicates, oxalates, phosphates) in the plant material, whereas the acid-insoluble ash corresponds to the content of silica and calcium oxalate[21]. The consumption of acid-insoluble ash must be limited since it contributes to renal oxalate deposition (Chaplin 1977). The ash levels of the HE-Es were lower than in leaf extracts of *Kalanchoe pinnata* (total ash=10.2 % and acid-insoluble ash=6.8 %)[22] and in the range of values of medicinal-plant powders of Western Himalaya (total ash=2.7-12.2 % and acid-insoluble ash=0.5-2.3 %)[23] and Nigeria (total ash=11.0-18.7 % and acid-insoluble ash=1.0-8.4 %)[24]. *E. subrigida* powder and HE-Es showed low moisture (Table 3), decreasing the risk of chemical and microbiological degradation; however, the HE-Es was hygroscopic and must be stored hermetically in refrigeration[13,25].

The organoleptic and physicochemical HE-Es properties could be useful as HE-Es' quality parameters (identity and purity) and to detect adulteration and inadequate manipulation of the plant materials[13,21,25]. The toxicological analyses showed a low total count of bacteria and fungi (Table 3) and absence of pathogenic bacteria (*Salmonella* spp., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*) and the analyzed toxins (B1, B2, G1, and G2). These values were within the norm, suggesting that HE-Es consumption is safe[13,25]. HE-Es showed no bitterness, no hemolysis, no astringency, and low content of tannins and foaming index (Table 4).

TABLE 3: QUALITY CONTROL PARAMETERS OF *Echeveria subrigida* FLOUR AND ITS HYDROALCOHOLIC EXTRACT (HE-Es)

Parameter	Flour	HE-Es
Organoleptic		
Color[1]	Opaque light green	Mustard
	(L=68.2, a=-10.35, b=20.48)	(L=46.47, a=3.29, b=14.2)
Particle size (mm)	0.4	0.4
Texture	Granular/soft	Granular/hard
Odor (strength/type)	Distinct/aromatic	Strong/fruity
Physicochemical[2]		
Ashes (%)		
Total	15.02±0.4	3.44±0.3
Acid insoluble	1.40±0.1	2.01±0.02
Water soluble	11.99±0.19	3.24±0.23
Weight loss by drying (%)	3.95±0.2	7.32±0.2
Toxicological (colonies/g)		
Bacteria	150	0
Fungus	8	0

Note: [1]: Color parameters in parenthesis were determined with a Minolta colorimeter and [2]: Values are the mean±SD of three determinations

TABLE 4: PHARMACOLOGICAL PARAMETERS OF THE HYDROALCOHOLIC EXTRACT OF *Echeveria subrigida* (HE-Es)

Parameter	HE-Es[1]	Control
Bitterness	0 units/g	Quinine hydrochloride (2000 units/g)
Hemolysis	0 %	Triton X100 (100 %)
Astringency (tannins)[2]	1.9±0.4 mg EC/g HE-Es	5 mg/mL Catechin
Swelling index	0 ml	-
Foaming index	≤100	-

Note: [1]: Values are the results of three determinations and [2]: mean±S.D

These parameters complied with norms established for natural products[13]; thus, it is suggested that HE-Es consumption will not induce adverse effects such as hemotoxicity[13,15]. The detection of tannins in HE-Es (1.9±0.4 mg CE/g) (astringency) agreed with its previous identification in the methanol extract of *E. subrigida*[10,12]; these compounds and polyphenols have also been identified in other Crassulaceae plants[26]. Consumption of tannins is associated with positive effects in human health (anti-inflammatory, antioxidant, anticonvulsant, antitumoral)[27], and the levels of these compounds in the HE-Es could contribute to its biological properties. On the other hand, the null HE-Es' swelling index suggested the absence of mucilaginous substances[13]. Besides, the HE-Es' foaming index was lower than 100, corresponding to low content of saponins and absence of hemolytic activity. The absence of saponins in the methanol extract of *E. subrigida*[10] supported our results.

The IC_{50} value for the α-glucosidase inhibitory activity of HE-Es (52.9±2.40 µg/ml) was lower than that of acarbose (858.3±35.49 µg/ml), showing a Coefficient Relative to Acarbose (RC_{Aca}) of 0.062. Several phenolics show low RC_{Aca} values (0.00944-0.2), being remarkably active quercetin and epigallocatechin gallate, and authors suggest that extracts/supplements containing these compounds could be useful in DM2 treatment[28,29].

HE-Es was a reversible inhibitor of α-glucosidase; the slope of the graph [p-NP] *vs.* time in the presence of inhibitor was lower than that in control (without inhibitor) and decreased with the increment of HE-Es concentration. The Lineweaver-Burk analysis showed that the HE-Es was a mixed inhibitor inducing variations in the V_{max} and K_m parameters[29]. In the reactions with inhibitor (HE-Es), the V_{max} (mM/min) values were 0.327 (50 µg/ml) and 0.156 (100 µg/ml), and the K_m (mM) values were 1.293 (50 µg/ml) and 0.866 (100 µg/ml); the values of both parameters were lower than those of α-glucosidase without inhibitor (V_{max}=0.504 mM/min; K_m=1.745 mM). Mixed inhibition has been reported for quercetin, Q3G, and rutin[30], as well as for tannins (epicatechin gallate and type A trimmers of epicatechin-(4β,8)-epicatechingallate)[4,31]. I3G is a less active inhibitor of α-glucosidase[4] and its inhibitory kinetics has not been reported; however, I3G showed mixed inhibition of tyrosinase[32].

The blood glucose levels in normoglycemic mice treated with HE-Es, 50 and 100 mg/kg b.w., induced a non-significant hypoglycemic effect ($p>0.05$) (fig. 2); probably, these doses are too low to overcome the physiological contra-regulatory mechanisms[33]. On the other hand, the treatment with 200 mg/kg b.w. of HE-Es induced a hypoglycemic effect after 3 h ($p<0.05$), which was similar to that produced by glibenclamide (10 mg/kg b.w.) ($p>0.05$) (fig. 2). The curves of hypoglycemic activities of the treatments HE-Es (200 mg/kg b.w.) and glibenclamide (10 mg/kg b.w.) showed similar decreasing tendencies and reached similar values at 5 h (fig. 2): the reductions in the blood glucose levels of treated mice were 49.1 % for HE-Es (200 mg/kg b.w.), 52 % for glibenclamide, and 25 % for the control. Thus, the HE-Es' hypoglycemic-effect was not considered severe in normoglycemic mice, similar results have been reported with other plant extracts[18]; consequently, phytochemicals of *E. subrigida* have similar efficacy to glibenclamide. The glibenclamide induces β-pancreatic cells to release insulin and

decreases liver gluconeogenesis[34]. In this regard, flavonoids (quercetin, isorhamnetin, tannins) also stimulate insulin secretion or trigger an insulin-like effect[18,35,36]. Thus, the HE-Es' hypoglycemic-effect could be associated with its content of flavonoids I3G and Q3G, but further studies are needed to support this hypothesis.

HE-Es induced antihyperglycemic activity in Balb-C mice, the oral tolerance curves were similar to that of acarbose (fig. 3). The HE-Es treatments avoid the increase in blood glucose levels at 30 min ($p<0.05$), the level of control mice increased an average of 90.8±7.9 mg/dl, but this effect was significantly reduced ($p<0.05$) in HE-Es treated mice: 77.2±9.5 mg/dl (50 mg/kg b.w.), 49.2±8.7 mg/dl (100 mg/kg b.w.), and 31.4±13.2 mg/dl (200 mg/kg b.w.) (fig. 3). Mice treated with acarbose increased the blood glucose an average of 20.7±12.2 mg/dl. This pattern of values could suggest the mechanism of action of HE-Es, HE-Es was a potent inhibitor of α-glucosidase and could induce a quick decrease in the postprandial hyperglycemia[37]. Strong inhibitory activity *in vitro* was also registered for the methanol extract of *E. subrigida* (IC_{50}=25.21 µg/ml)[10], an activity that is associated with the content of flavonoids (I3G, Q3G, and proanthocyanidins)[12].

Comparing with the complete glycemic response (Area under curve) of treated control animals, a significant percent reduction in glucose ($p<0.05$) was only observed in mice treated with acarbose (10 mg/kg b.w., 19.87 %) and HE-Es at 100 mg/kg b.w. (29.32 %) and 200 mg/kg b.w. (28.99 %) (fig. 4). These glycemic responses of mice were similar to that reported for quercetin (300 mg/kg b.w.), which decreased 30.3 % the postprandial glucose level on Wistar rats treated with maltose (2 g/kg b.w.)[38]. The stem extract of *Acalypha indica* Linn (600 mg/kg b.w.) induces a 52 % decrease in rats' blood glucose level[39]. Thus, the standardized HE-Es could be considered as an effective antihyperglycemic agent based on the tested concentrations. In this regard, different standardized extracts have shown positive effects on glucose metabolism. The *Ficus deltoidea* extract, standardized in the content of C-glycosylflavones, shows antidiabetic effect on rats with Streptozotocin (STZ) induced diabetes[40]. The extract of *Ficus carica* standardized in abscisic acid improves the glycemic and insulinemic responses of healthy human adults[41].

Different mechanisms could mediate the hypoglycemic and antihyperglycemic activities of HE-Es, inhibition of intestinal glucose absorption, inhibition of liver gluconeogenesis, stimulation of tissue glycolysis, stimulation of insulin release, and induction of insulin-like effects[35,36]. However, further studies must be carried out to demonstrate how the HE-Es is regulating the glucose levels.

The developed HPLC method to standardize the HE-Es complies with established norms to quantitate the marker compound (isorhamnetin-3-glucoside, I3G). The standardized HE-Es extract ([I3G]=4.81 mg/g) showed hypoglycemic and antihyperglycemic activities, as well as physicochemical, pharmacological, and toxicological characteristics that support its antidiabetic potential, stability, and innocuity. The standardized HE-Es is a potential antidiabetic phytopharmaceutical.

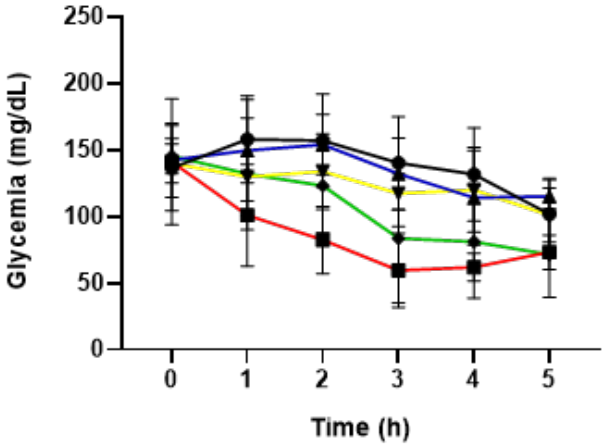

Fig. 2: Hypoglycemic activity of the hydroalcoholic extract of *Echeveria subrigida* (HE-Es) on normoglycemic Mice. Data are the mean±SE, n=6; *: Significant differences with respect to the control at the same time ($p<0.05$)
Note: ( ): Control; ( ): Glibenclamide 10 mg/kg; ( ): HE-Es 50 mg/kg; ( ): HE-Es 100 mg/kg and ( ): HE-Es 200 mg/kg

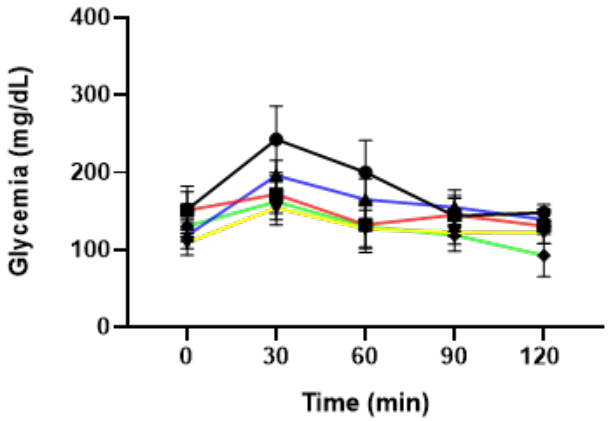

Fig. 3: Effect of the treatment with the hydroalcoholic extract of *Echeveria subrigida* (HE-Es) on the glycemic response curve of normoglycemic mice after sucrose administration (2 g/kg b.w.). Data are the mean±SE, n=6; *: Significant differences with respect to the control at the same time (p<0.05)
Note: ( ): Control; ( ): Acarbose 10 mg/kg; ( ): HE-Es 50 mg/kg; ( ): HE-Es 100 mg/kg and ( ): HE-Es 200 mg/kg

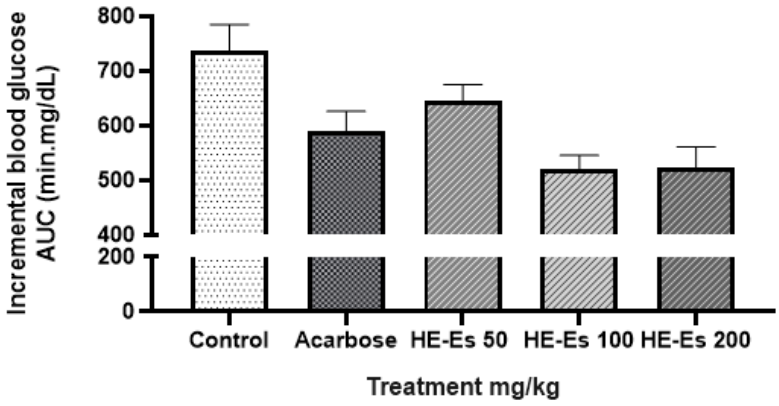

Fig. 4: Effect of the treatment with the hydroalcoholic extract of *Echeveria subrigida* (HE-Es) on the incremental area under the curve 0-120 min of normoglycemic mice after sucrose administration (2 g/kg b.w.). Data are the mean±S.E., n=6; *: Significant differences with respect to the control (p<0.05)

Author's contribution:

B. Heredia-Mercado and F. Delgado-Vargas contributed equally to this work

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Conflict of interest:

The authors declared no conflict of interest.

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