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Original Article

Antidiabetic Potential and Chronic Toxicity of Hydroalcoholic Extract of *Echeveria subrigida* Leaves

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ABSTRACT

Background: *Echeveria subrigida* extracts have biological activities of human health importance. However, the *in vivo* effects on glucose levels and chronic toxicity are unknown. **Objectives:** To analyse the *in vivo* hypoglycemic and antihyperglycemic effects (50, 100, and 200 mg/kg b.w.) and chronic toxicity (1000 mg/kg b.w. for 270 days) of the hydroalcoholic extract of *E. subrigida* (HE–Es) in BALB/c mice. **Materials and Methods:** The HE–Es was analysed by HPLC. Glucose levels were measured to establish the effects on glycemia. Different parameters were registered in the toxicity assay: *e.g.*, feed consumption, appearance/behaviour, biochemical and haematological parameters and liver and kidney histologies. **Results:** Quercetin–3-*O*-β-glucoside and isorhamnetin–3-*O*-β-glucoside were the main flavonoids in the HE–Es. Glycemia was reduced by the HE–Es (200 mg/kg b.w., 49.1%) and glibenclamide (10 mg/kg b.w., 52%) treatments. Comparing the antihyperglycemic activities, similar reductions were found between HE–Es (100 mg/kg b.w., 29.32%; 200 mg/kg, 28.99%) and acarbose (10 mg/kg b.w., 19.87%) treatments. On the other hand, the HE–Es was innocuous in mice (LD_{50} > 1000 mg/kg b.w.), and the results suggested that HE–Es had adaptogenic, and immunostimulant activities. The hepatic and renal histologies were normal; however, male mice showed level zero steatosis that disappeared in the HE–Es satellite after treatment withdrawal. **Conclusion:** This study reports for the first time the *in vivo* effects on blood glucose levels of HE–Es and chronic toxicity of a Crassulaceae plant, supporting the antidiabetic potential and safety of HE–Es. Future studies must corroborate the effects of HE–Es in humans, allowing its use in high-value formulations.

Keywords: Crassulaceae family, Safety, Plant natural extract, Hypoglycemia, Antihyperglycemia.

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INTRODUCTION

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

Traditional medicine comprises different cultures' knowledge, abilities and practises to maintain health and prevent, diagnose and treat physical and mental diseases. Different plant natural products contain compounds with hypoglycemic properties (*e.g.*, flavonoids, alkaloids, steroids, peptides) and potential to be developed as new antidiabetic drugs. Flavonoids (*e.g.*, flavones, and flavonols) are potent inhibitors of α-glucosidase.[4] Thus, medicinal plants are booming due to the high cost of allopathic drugs and the global trend toward using natural and organic products as preventive health agents or drug alternatives.[5] First, however, traditional plant remedies must be scientifically evaluated to assure their safety and efficacy.

The *Echeveria* genus (Crassulaceae) includes many species distributed from Texas (US) to Argentina. Most *Echeveria* spp. are endemic to Mexico and traditionally employed as ornamental or medicinal plants to treat bacterial infections, gastrointestinal disorders, fever, toothache, and scorpion bites, among other uses.[6] Extracts of *E. subrigida* leaves have shown relevant

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biological activities for humans: *e.g.*, adaptogenic, antioxidant, antimicrobial, inhibitory of α-glucosidase (IG), antimutagenic, and immunomodulatory.[7–9] Remarkably, the IG was associated with flavonoids and tannins content,[8] these studies support the potential of *E. subrigida* extracts to treat DM2, but the *in vivo* effect on glycemia and their safety have not been established. On the other hand, most toxicity studies of Crassulaceae plants have been conducted in acute toxicity models,[10–15] and there are no reports of chronic toxicity. Furthermore, the HE–Es does not show acute toxicity in mice.[9] Thus, the aims of this study were to evaluate in normoglycemic BALB/c mice the effects on the basal and postprandial glucose levels and chronic toxicity of the hydroalcoholic extract of *Echeveria subrigida* leaves.

MATERIALS AND METHODS

Plant material

Leaves of *E. subrigida* (B. L. Rob. and Seaton) Rose were collected near to "El Palmito" town, Concordia, Sinaloa, Mexico (2000 m.a.s.l.; lat. N. 23° 34' 06", long. W. 105° 50' 53"). Dr. Rito Vega–Aviña from the School of Agronomy, Autonomous University of Sinaloa, identified the plant corresponding with a specimen with voucher number 11742 in the herbarium. *Echeveria subrigida* leaves were washed, drained at room temperature (25°C), cut into small pieces, frozen at -80°C, and freeze dried. The plant material was milled to obtain a powder that passed through a 40 mesh.

Animals

BALB/c mice (Bioinvert, SA de CV, Mexico) were maintained at 24 ± 2°C, 50% humidity, 12 hr light–dark cycles, and provided with water, and feed (Nutricubos, Nutrimentos Purina S.A. de C.V., Mexico) *ad libitum*. The study was carried out according to the Mexican Official Norm NOM–062-ZOO–1999 and the guide for the care and use of laboratory animals.[16]

Reagents and solvents

Solvents were HPLC grade (TEDIA, US), and reagents were Sigma Aldrich (St. Louis, MO, US).

Hydroalcoholic extract preparation

The *E. subrigida* powder (100 g) and 80% ethanol were mixed (1:10 w/v) and agitated (150 rpm/ 1 day). The hydroalcoholic phase was recovered by filtration (Whatman filter paper No. 2). The residue was extracted twice, and the hydroalcoholic phases were mixed, concentrated in a rota–evaporator (BÜCHI Labortechnick AG, Switzerland) at 40°C, dried in vacuum–oven (Prendo SS–250, Mexico) at 40°C for 24 hr, and freeze dried. The residue was the hydroalcoholic extract of *E. subrigida* (HE–Es); it was stored at −20°C under N_2 atmosphere and protected from light until use.

HPLC analysis

The HE–Es was dissolved in methanol:water (9:1) (5 mg/mL) and filtered prior to the analysis. The HPLC analyses were performed using an HPLC–DAD ACCELA (Thermo Scientific, US) provided with an ACE EXCEL C_{18}–Amide column (150 × 30 mm × 3 μm) (Advanced Chromatography Technologies, UK). The mobile phase consisted of 1% formic acid (A) and acetonitrile (B): 0.5% B, linear gradient to 16% B in 4 min, linear gradient to 60% B in 17 min, and isocratic 5 min. The separation conditions were as follows: running time 35 min, flow 0.3 mL/min, injection volume 15 μL, and detection at 280, 320, and 350 nm. The identity of the main peaks was corroborated based on spectroscopic and spectrometric data in our previous research.[8] Flavonoids were measured as equivalents of quercetin (EQ) or isorhamnetin (EI) per gram of extract (mg E/g HE–Es) by employing the corresponding calibration curves (0–40 μg/mL).

Effect of HE–Es on glycemia of normoglycemic mice

Hypoglycemic activity

The hypoglycemic activity was determined as reported,[17] with some modifications. Five groups (n = 6) of BALB/c mice were formed: group I, 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 6 hr fasting; immediately, treatments were administered orally, and glucose levels were measured every hour for 5 hr. 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 (oral glucose tolerance test)

Oral glucose tolerance test was carried out as previously described,[18] 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 hr 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/dL of blood and as the incremental area under the blood glucose response curve (iAUC) (mg•min/dL).

Chronic toxicity evaluation

Chronic toxicity was evaluated following the recommendations of the OECD.[19] BALB/c mice were divided into three groups of each sex ($n = 10$): group I (control, saline solution), group II (HE–Es, 1000 mg/kg b.w), and group III satellite (HE–Es, 1000 mg/kg b.w). The satellite group was used to determine the reversibility of the possible toxic effects after the treatment was ended. The doses or vehicles were orally administered daily for 270 days.

Along the treatment were registered changes in skin, hair, eyes, mucosa membranes, secretions, and excretions, and autonomic activity (*e.g.*, tearing, piloerection, pupil size, unusual respiratory pattern). The changes in walking patterns, posture, manipulation response, and unusual behaviours (*e.g.*, excessive grooming, repetitive circular movements, auto-mutilation) were also described. The aggressivity was determined, registering the increment or decrease in the number of lesions, latency time of attacks, and the number of fights or agonistic encounters.[20] The observations were carried out during the first 12 hr after treatment and then daily.

After 270 days, mice from control and HE–Es groups were fasted overnight, anaesthetised with pentobarbital, and sacrificed. Blood was obtained by cardiac punction and put into two tubes, one with anticoagulant (EDTA) for the haematological analysis, and the other without anticoagulant for the biochemical analysis. Animals were dissected, and the organs were recovered and examined macroscopically and microscopically.

The mice of the satellite group were maintained for other 28 days without HE–Es treatment and then processed as indicated in this section.

Feed consumption, weight gain, and relative weight of organs

The feed consumption was evaluated daily as the difference between the feed supplied and consumed. The provided feed was adjusted weekly according to the animal requirements.

The weight gain (%) was determined weekly with the following formula:

$$\text{Weight gain (\%)} = \left(\frac{\text{(final weight-initial weight)}}{\text{initial weight}}\right) \times 100$$

The feed consumption and weight gain data were averaged per month to compare the treatments.

The relative weight of organs (%) was determined as follows:

$$\text{Relative weight of organs (\%)} = \left(\frac{\text{(organ weight)}}{\text{body weight}}\right) \times 100$$

Evaluation of the hematologic parameters

The hematologic parameters were measured with an automatic analyzer (Beckman Coulter Act 5 Diff, Switzerland). The differential counting of white cells was carried out by blood smear and Wright's stain (blue methylene and eosin).[21]

Evaluation of biochemical parameters

Blood samples without anticoagulant were centrifuged (2500 rpm/ 5 min) to obtain the serum and evaluate the following components: glucose, hepatic function tests (aspartate aminotransferase AST and alanine aminotransferase ALT), total proteins (TP), total (TB) and direct bilirubin (DB), and kidney function tests (urea and creatinine). Colorimetric diagnostic kits (Pointe Scientific INC, US) were used for all determinations.

Histological processing of liver and kidney

Transversal liver slices and longitudinal kidney slices of 5 mm thickness were prepared. Slices were fixed with 10% formalin, dehydrated in a Leica Tissue Processor TP1020 Histokinette (Leica Microsystems, Germany) with aqueous solutions of increasing ethanol concentration (70%, 95%, and absolute ethanol), cleared with xylene, and embedded in Paraplast wax. The liver and kidney embedded tissues were cut in 6 µm–thick sections using a Leica RM2125 rotary microtome. The sections were stained with Harris' hematoxylin and eosin Y (H&E) and Masson trichrome stain, mounted in Entellan synthetic resin (Merck, Germany), observed with an optical microscope Primo Star (Carl Zeiss, Germany), and photographed using the 10x, and 40x objective lens. The images were processed with the Zen2 Blue Edition software (Zeiss, Germany). The steatosis level was evaluated with the S histologic punctuation system of the Clinical Research Network on Non-alcoholic Fatty Liver Disease (NAFLD).[22]

Statistical analysis

Data were analysed by one-way analysis of variance using the GraphPad Prism 8 software (Intuitive Software for Science, San Diego, CA, US). The hypoglycemic and antihyperglycemic activities were analysed by one-way ANOVA 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. Feed consumption, body weight, organ weight, and hematologic and biochemical parameters were the response variables. The Fisher test was used for mean comparisons ($p < 0.05$). Experiments were carried out at least in triplicate.

RESULTS

HPLC analysis

The HPLC analysis showed two prominent flavonoid peaks: quercetin–3-*O*-β-glucoside (1) and isorhamnetin–3-*O*-β-glucoside (2) (Figure 1), containing 3.77±0.171 mg EQ/g HE–Es and 4.21±0.093 mg EI/g HE–Es.

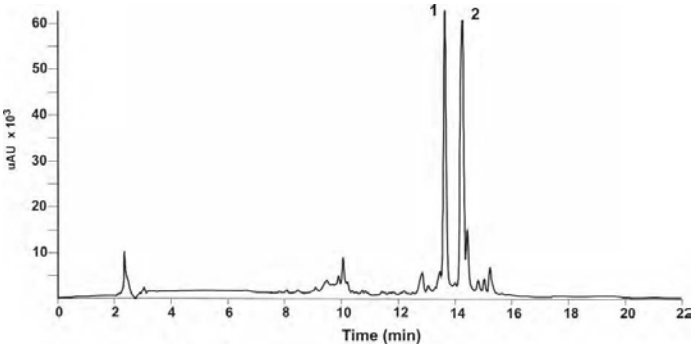

Figure 1: HPLC–DAD chromatogram (total scan) of the hydroalcoholic extract of *Echeveria subrigida* (HE–Es). The prominent peaks were identified as quercetin–3-*O*-β-glucoside (1) and isorhamnetin–3-*O*-β-glucoside (2).

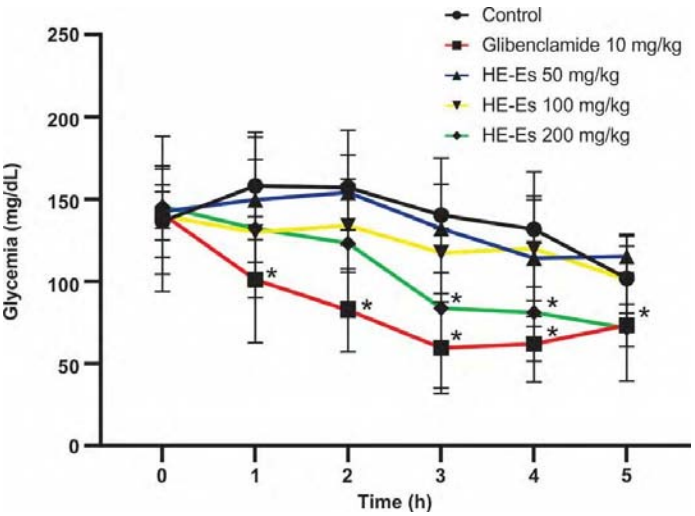

Figure 2: Hypoglycemic activity of the hydroalcoholic extract of *Echeveria subrigida* (HE–Es) on normoglycemic mice. Data are the mean ± S.E., $n = 6$. *Value shows significant difference compared with control value at the same time ($p < 0.05$).

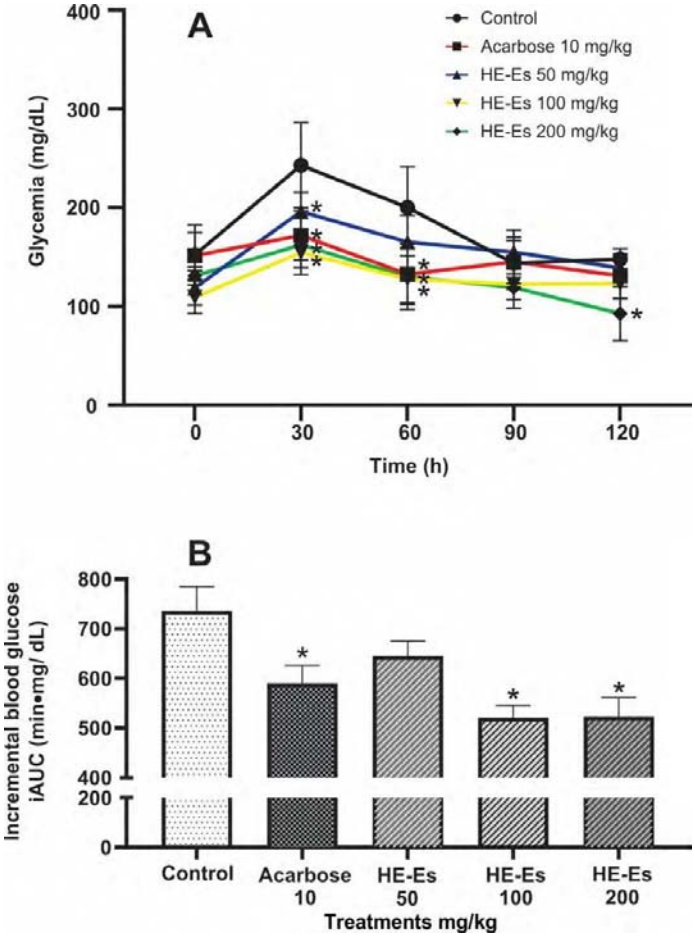

Figure 3: Effect of the hydroalcoholic extract of *Echeveria subrigida* (HE–Es) in the glycemia of normoglycemic mice after sucrose administration (2 g/kg b.w.): (A) response curve and (B) incremental area under the curve 0–120 min (iAUC). Data are the mean ± S.E., $n = 6$. *Value shows significant difference compared with control value at the same time (A) or with that of the control group (B) ($p < 0.05$).

Effect of HE–Es on glycemia of normoglycemic mice

Hypoglycemic activity

The HE–Es treatments (50 and 100 mg/kg b.w.) in normoglycemic mice induced a non-significant hypoglycemic effect ($p>0.05$) (Figure 2). On the other hand, the HE–Es treatment with 200 mg/kg b.w. induced a hypoglycemic effect after 3 hr ($p< 0.05$), which was similar to that produced by glibenclamide (10 mg/kg b.w.) ($p>0.05$) (Figure 2). The hypoglycemic–activity curves 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 hr (Figure 2): the reductions on the blood glucose levels in treated mice were 49.1% for HE–Es (200 mg/kg b.w.), 52% for glibenclamide, and 25% for the control.

Antihyperglycemic activity (oral glucose tolerance test)

The HE–Es induced antihyperglycemic activity in BALB/c mice, and the oral tolerance curves were similar to that of acarbose (Figure 3A). The HE–Es treatments diminish the increase in blood glucose levels at 30 min ($p< 0.05$). Compared with the glucose level in control mice (90.8 ± 7.9 mg/dL), the values in HE–Es treated mice were significantly lower ($p< 0.05$): 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.) (Figure 3A). In contrast the value for the acarbose treated mice was 20.7 ± 12.2 mg/dL.

Compared with the complete glycemic response of control mice, a significant reduction in glucose ($p< 0.05$) was 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%) (Figure 3B).

Chronic toxicity evaluation

Survival, behaviour, and clinical manifestations of mice treated with the hydroalcoholic extract of Echeveria subrigida (HE–Es)

All mice treated with HE–Es (1000 mg/kg b.w./day/270 days) survived. Besides, male mice showed yellowish hair associated with urine stains up to the first month of treatment, characteristic non-observed in the female mice; then, the colour gradually

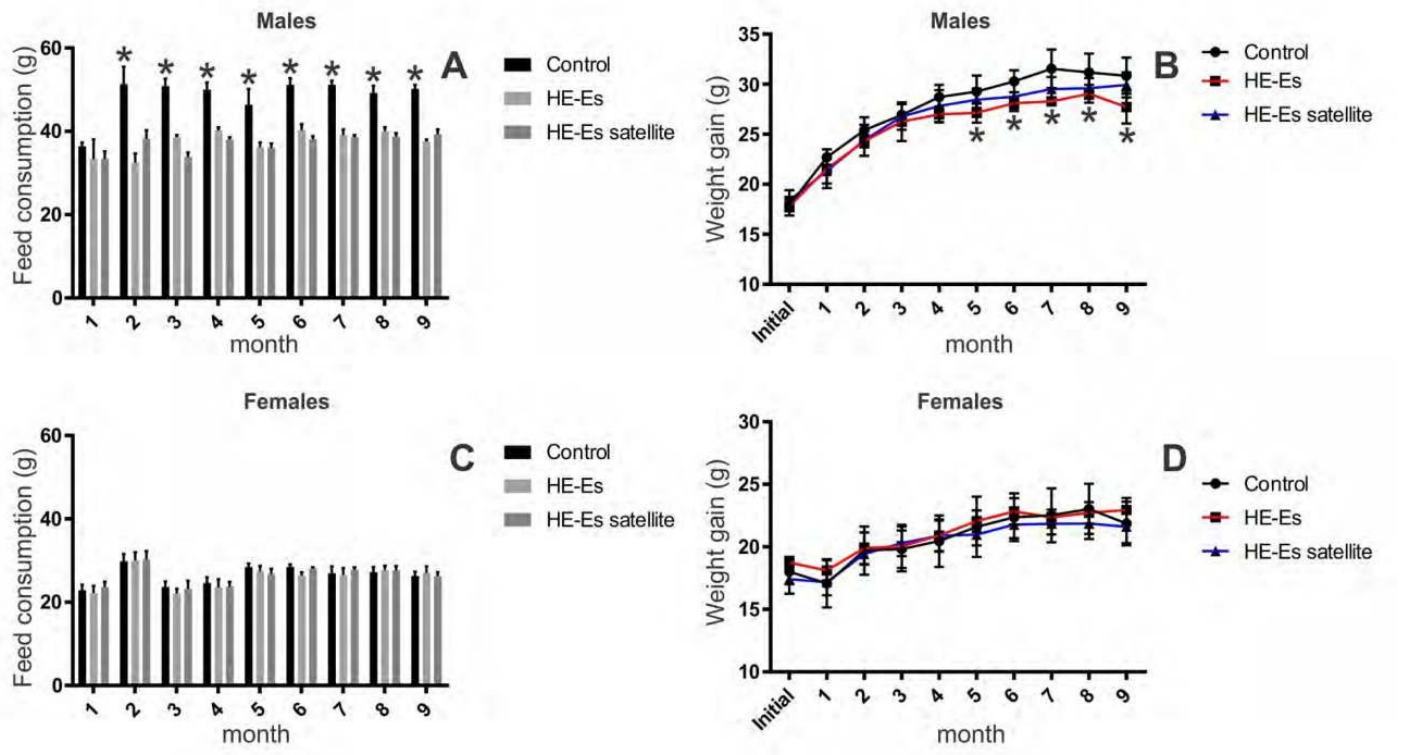

Figure 4: Effect of the chronic administration (270 days) of the hydroalcoholic extract of *Echeveria subrigida* (HE–Es) (1000 mg/kg of b.w./ day) on feed consumption and weight of mice: males (A and B) and females (C and D). Values are the mean ± standard error of six mice per group. *Indicate significant difference ($p < 0.05$) between mice of the control group and those treated with HE–Es.

disappeared within fifteen days. On the other hand, female mice of the control group developed alopecia in the third month of treatment, losing part of the hair of the head and whiskers and recovering after one month; the females in groups HE–Es and HE–Es satellite did not show this symptom.

Mice showed normal skin and eyes; no secretions or excretions of the mucosa membranes were registered.

The mice's behaviour was similar among groups. Gait changes, tonic–clonic seizures, repetitive circular movements, and auto-mutilation were not observed. However, the male mice were more aggressive than the female mice, and this behaviour was more evident at the start of the treatment. Nevertheless, the treated male mice groups (HE–Es, and HE–Es satellite) were less aggressive than those untreated from the first week of dose administration until the end of the assay.

Feed consumption, weight gain, and relative weight of organs

Feed consumption of treated male mice (HE–Es and HE–Es satellite) was lower than that of untreated male mice throughout the study ($p< 0.05$) (Figure 4A). This feeding behaviour was reflected in the weight gain of male mice: the untreated animals showed higher body weight than the treated ones, reaching significant differences from the fifth month until the end of the experiment ($p< 0.05$) (Figure 4B and Table 1). The male mice of the HE–Es satellite group recovered weight after treatment withdrawal (Table 1). On the other hand, female mice of all groups showed similar feed consumption and weight ($p> 0.05$) (Figure 3C and 3D and Table 1). In this regard, variations in feed consumption or weight gain were not reflected in the relative weight of organs ($p> 0.05$) (Table 1).

Hematologic parameters

The haemoglobin and hematocrit values of HE–Es treated mice (both sexes) were similar to those of untreated mice ($p> 0.05$) (Table 2). In the differential white blood cell count, significant differences were only found for the HE–Es treated female mice, showing values increased for lymphocytes and decreased for neutrophils. However, the number of lymphocytes and neutrophils in female HE–Es satellite mice at the end of the treatment was similar to those of control mice, indicating the mice's recovery after withdrawing from the HE–Es treatment (Table 2).

Biochemical parameters

The HE–Es treated mice (male and female) showed lower glucose levels than those of control mice ($p< 0.05$), accordingly to the hypoglycemic HE–Es activity described in this paper (Table 3). In addition, all mice showed similar values of creatinine, total and direct bilirubin, AST and ALT, suggesting normal hepatic and renal function. However, the HE–Es treated females showed increased levels of TP ($p< 0.05$) (Table 3).

Table 1: Effect of the treatment for 270 days with the hydroalcoholic extract of *Echeveria subrigida* (HE–Es) (1000 mg/kg of b.w./ day) in the weight gain and relative weight of organs of mice.

Parameter	Treatments		
	Control	HE–Es	HE–Es satellite
Male			
Weight gain (%)	72.5 ± 7.72[a]	55.5 ±5.01[b]	63.0 ± 7.01[c]
Relative weight of organs			
Liver	5.55 ± 0.60	5.59 ± 0.58	5.48 ± 0.34
Kidney	1.05 ± 0.09	1.02 ± 0.13	1.04 ± 0.07
Heart	0.67 ± 0.07	0.64 ± 0.06	0.69 ± 0.35
Spleen	0.38 ± 0.02	0.40 ± 0.06	0.37 ± 0.06
Lung	1.00± 0.20	1.04 ± 0.37	0.96 ± 0.04
Testicle	0.39 ± 0.02	0.40 ± 0.04	0.37 ± 0.06
Female			
Weight gain (%)	21.5 ± 6.9	22.5 ± 7.6	19.1 ± 7.0
Relative weight of organs			
Liver	5.12 ± 0.27	4.87 ± 0.19	5.45 ± 0.26
Kidney	0.73 ± 0.03	0.75 ± 0.04	0.77 ± 0.05
Heart	0.56 ± 0.07	0.55 ± 0.07	0.61 ± 0.10
Spleen	0.42 ± 0.10	0.43 ± 0.01	0.46 ± 0.15
Lung	1.07 ± 0.15	1.16 ± 0.10	1.18 ± 0.16

Values are the media ± standard error of 6 mice per group. The values in rows do not show statistical differences ($p > 0.05$) but the weight gain of males ($p<0.05$), differences indicated by different superscript letters.

Hepatic and renal histological analysis

Hepatic tissue

The liver slices stained with H&E of all mice showed normal parenchyma and architecture characterised by hepatocyte cords, a low number of immune cells, viable hepatocytes of polyhedric form and nucleus in the centre, scarce apoptotic cells and an absence of necrotic cells (Figures 5 and 6). However, HE–Es treated male mice showed hepatocytes with fat vacuoles in the cytoplasm, corresponding with macrovesicular steatosis in about 5% of the area around the central veins (hepatic zone 3). In addition, the central and hepatic sinusoid veins of treated and untreated mice showed similar diameter and appearance and absence of infiltrated inflammatory cells. On the other hand, slices stained with the Masson trichrome stain of female and male mice showed normal distribution of collagen in the portal area and absence of collagen fibres, discarding the induction of fibrosis (Figure 7).

Renal tissue

The slices of renal tissues stained with H&E of female and male mice showed normal morphology and similar structural characteristics (Figure 8): glomerular and tubular, proximal and distal contoured tubules, Henle loop, collecting tubules, dense macula, mesangial cells and Bowman space. These characteristics suggest that HE–Es is innocuous for the kidney. Besides, the kidney slices stained with the Masson trichrome stain showed typical characteristics without signs of tubulointerstitial and glomerular fibrosis, discarding glomerulonephritis (Figure 9).

DISCUSSION

HPLC analysis of the hydroalcoholic extract of *E. subrigida*

The main flavonoids in the HE–Es (Figure 1) agreed with those in the methanolic extract of *E. subrigida*, which showed quercetin–3-*O*-β-glucoside (2.9 mg EQ/g) and isorhamnetin–3-*O*-β-glucoside (4.6 mg EI/g), compounds that were associated with the α-glucosidase inhibitory activity of the methanol extract.[7]

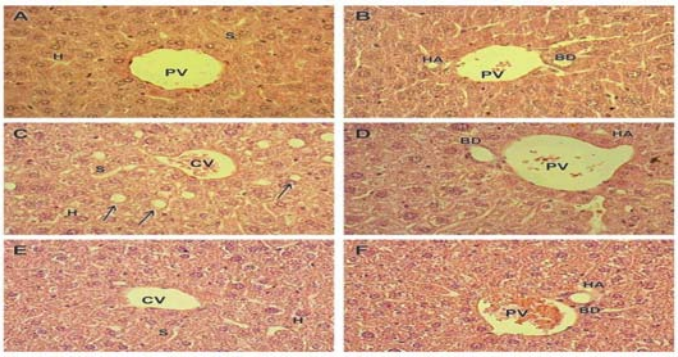

Figure 5: Effect of treatment for 270 days with the hydroalcoholic extract of *Echeveria subrigida* (HE-Es) (1000 mg/kg of b.w./day) on hepatic morphology of male mice: (A and B) negative control, saline solution; (C and D) HE-Es, HE-Es (1000 mg/kg/day); (E and F) HE- Es satellite, HE-Es (1000 mg/kg/day for 270 days + 28 days without treatment). CV, Central Vein; PV, Porta Vein; BD, Biliary Duct; HA, Hepatic Artery; H, Hepatocyte; S, Sinusoid. Arrows indicate macrovesicular steatosis around the central vein for the HE-Es group. Tissues were stained with hematoxylin and eosin (H&E), 40X magnification.

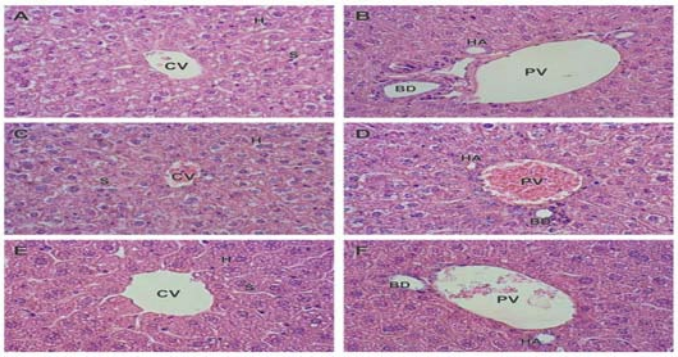

Figure 6: Effect of treatment for 270 days with the hydroalcoholic extract of *Echeveria subrigida* (HE-Es) (1000 mg/kg of b.w./day) on hepatic morphology of female mice: (A and B) negative control, saline solution; (C and D) HE-Es, HE-Es (1000 mg/kg/day); (E and F) HE-Es satellite, HE-Es (1000 mg/kg/day for 270 days + 28 days without treatment). CV, Central Vein; PV, Porta Vein; BD, Biliary Duct; HA, Hepatic Artery; H, Hepatocyte; S, Sinusoid. Tissues were stained with hematoxylin and eosin (H&E), 40X magnification.

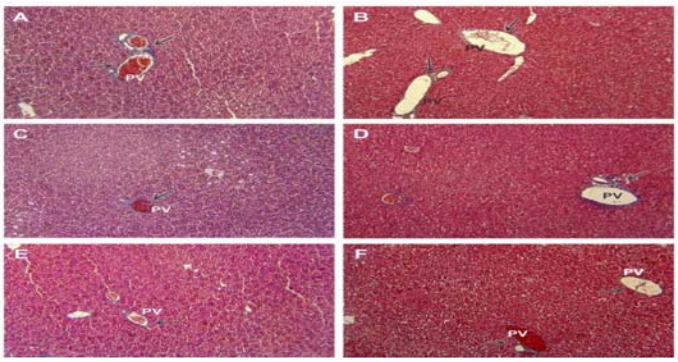

Figure 7: Effect of the administration of the hydroalcoholic extract of *Echeveria subrigida* (HE-Es) (1000 mg/kg of b.w./day) on the accumulation of fibrous tissue in the liver of male (left) and female (right) mice: (A and B) negative control, saline solution; (C and D) HE-Es, HE-Es (1000 mg/kg/day); (E and F) HE-Es satellite, HE-Es (1000 mg/kg/day for 270 days + 28 days without treatment). Arrows indicate presence of collagen in liver tissue (blue) mainly in the porta vein (PV) structure. Tissues were stained with Masson's trichrome, 10X magnification.

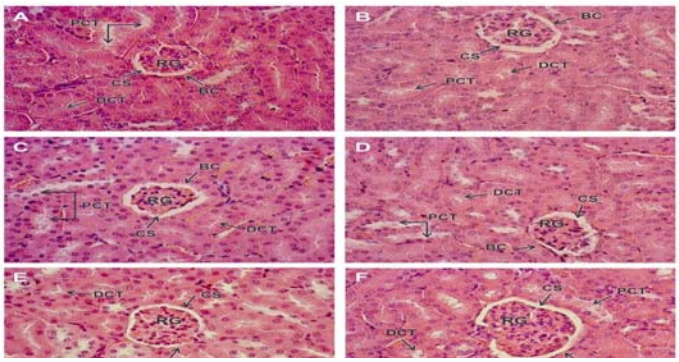

Figure 8: Effect of the administration of the hydroalcoholic extract of *Echeveria subrigida* (HE-Es) (1000 mg/kg of b.w./ day) on renal morphology of male (left) and female (right) mice: (A and B) negative control, saline solution; (C and D) HE-Es, HE-Es (1000 mg/kg/day); (E and F) HE-Es satellite, HE-Es (1000 mg/kg/day for 270 days + 28 days without treatment). RG, Renal Glomeruli; DCT, Distal Convoluted Tubule; PCT, Proximal Convoluted Tubule; CS, Corpuscular Space; BC, Bowmann's Capsule. Tissues were stained with hematoxylin and eosin (H&E), 40X magnification..

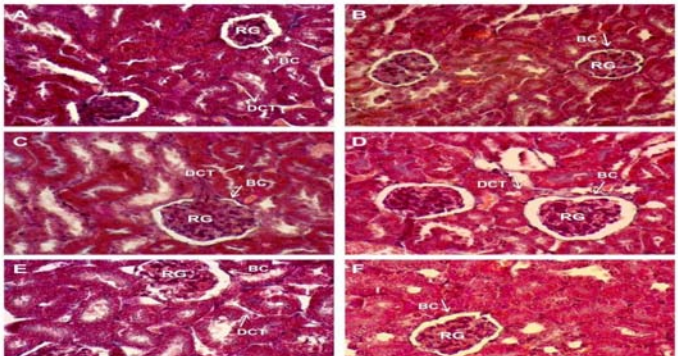

Figure 9: Effect of the administration of the hydroalcoholic extract of *Echeveria subrigida* (HE-Es) (1000 mg/kg of b.w./day) on accumulation of fibrous tissue in the glomerular and tubulo-interstitial areas of male (left) and female (right) mice: (A and B) negative control, saline solution; (C and D) HE-Es, HE-Es (1000 mg/kg/day); (E and F) HE-Es satellite, HE- Es (1000 mg/kg/day for 270 days + 28 days without treatment). RG, Renal Glomeruli. Arrows indicate presence of collagen in renal tissue (blue) mainly in the Distal Convoluted Tubule (DCT) and Bowmann's Capsule (BC) structure. Tissues were stained with Masson's trichrome, 40X magnification.

Table 2: Haemoglobin, hematocrit, and differential white blood cell count (WBC) of mice treated for 270 days with the hydroalcoholic extract of *Echeveria subrigida* (HE–Es) (1000 mg/kg of b.w./ day).

Type of cell (%)	Treatment		
	Control	EH–Es	EH–Es satellite
Male			
Haemoglobin (g/dL)	15.27 ± 1.1	14.07 ± 1.2	15.95 ± 0.7
Hematocrit (%)	47.15 ± 3.3	43.72 ± 3.7	49.59 ± 2.4
Differential WBC (%)			
Neutrophils	31.7± 5.10[a]	27.4 ± 3.21[a]	30.8 ± 3.41[a]
Lymphocytes	65.1 ± 1.21[a]	70.8 ± 2.40[a]	67.8 ± 2.71[a]
Monocytes	0.7 ± 0.30 [a]	1 ± 0.40[a]	1 ± 0.70[a]
Eosinophils	0.1 ± 0.00[a]	0.1 ± 0.00[a]	0.1 ± 0.00[a]
Basophils	2 ± 0.10[a]	1.1 ± 0.10[a]	1.1 ± 0.10[a]
Female			
Haemoglobin (g/dL)	15.16 ± 0.8	15.22 ± 0.6	16.05 ± 1.6
Hematocrit (%)	46.64 ± 2.5	47.23 ± 1.9	49.92 ± 5.0
Differential WBC (%)			
Neutrophils	24 ± 4.30[a]	11.1 ± 2.15[b]	18.5 ± 6.20[a]
Lymphocytes	75.5 ± 4.60[a]	87.6 ± 3.21[b]	79.5 ± 2.71[a]
Monocytes	0 ± 0.00[a]	1 ± 0.00[a]	0.3 ± 0.00[a]
Eosinophils	0 ± 0.00[a]	0 ± 0.00[a]	0.3 ± 0.00[a]
Basophils	1 ± 0.00[a]	0 ± 0.00[a]	0 ± 0.00[a]

Values are the media ± standard error of 6 mice per group. Different superscript letters in the same row indicate significant differences ($p< 0.05$).

Effect of HE–Es on glycemia of normoglycemic mice

Hypoglycemic activity

The non-significant hypoglycemic effect in normoglycemic mice treated with HE–Es (50 and 100 mg/kg b.w.) (Figure 2) was not considered severe and similar results have been reported with other plant extracts;[17] probably, these doses are too low to overcome the physiological contra–regulatory mechanisms.[23] The phytochemicals in HE–Es had similar efficacy than glibenclamide, drug which induces β-pancreatic cells to release insulin and decreases liver gluconeogenesis.[24] In this regard, flavonoids (*e.g.*, quercetin, isorhamnetin, tannins) stimulate insulin secretion or trigger an insulin–like effect.[17,25,26] Thus, the HE–Es' hypoglycemic effect could be associated with its content of flavonoids, but further studies are needed to support this hypothesis.

Antihyperglycemic activity (oral glucose tolerance test)

BALB/c mice treated with HE–Es and acarbose showed similar oral tolerance curves (Figure 3A). Compared with the glucose levels of control mice at 30 min (90.8 ± 7.9 mg/dL), the values for the HE–Es treated mice were dose–dependent and reduced up to 31.4 ± 13.2 mg/dL at 200 mg/kg b.w. (Figure 3A). The value for the acarbose–treated mice was 20.7 ± 12.2 mg/dL. This pattern of values suggests the HE–Es mechanism of action: HE–Es could be a potent α-glucosidase inhibitor and induce a rapid decrease in postprandial hyperglycemia.[27] A hypothesis supported by the strong α-glucosidase inhibitory activity registered for the methanol extract of *E. subrigida* (IC_{50} = 25.21 μg/mL),[8] an activity that is associated with the content of flavonoids (I3G, Q3G, and proanthocyanidins).[7]

The complete glycemic response in mice showed significant glucose level reduction in those 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%) (Figure 3B). 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 in Wistar rats treated with maltose (2 g/kg b.w.).[28] The stem extract of *Acalypha indica* Linn. (600 mg/kg b.w.) induces a 52% decrease in the blood glucose level of rats.[29] Thus, standardised HE–Es could be considered an effective antihyperglycemic agent. In this regard, different standardised extracts have positively affected glucose metabolism. The *Ficus deltoidea* extract, standardised in the content of *C*–glycosylflavones, shows antidiabetic effect on rats with streptozotocin (STZ) induced diabetes.[30] The extract of

Table 3: Blood chemistry of mice treated for 270 days with the hydroalcoholic extract of *Echeveria subrigida* (HE–Es) (1000 mg/kg of b.w./ day).

Parameter	Treatment		
	Control	EH–Es	EH–Es satellite
Male			
Glucose (mg/dL)	113 ± 23.61[a]	69.3 ± 3.00[b]	136 ± 12.10[a]
Urea (mg/dL)	40 ± 5.20[a]	40 ± 6.00[a]	39 ± 6.21[a]
Creatinine (mg/dL)	1.0 ± 0.10[a]	1.07 ± 0.10[a]	1.02 ± 0.10[a]
Total bilirubin (mg/dL)	0.82 ± 0.11[a]	0.99 ± 0.41[a]	1.04 ± 0.30[a]
Direct bilirubin (mg/dL)	0.49 ± 0.10[a]	0.48 ± 0.10[a]	0.51 ± 0.10[a]
AST (U/L)	74.1 ± 18.50[a]	54.5 ± 32.00[a]	87.7 ± 8.91[a]
ALT (U/L)	60 ± 32.61[a]	75 ± 26.92[a]	70 ± 32.91[a]
Total proteins (g/dL)	5.3 ± 0.61[a]	4.3 ± 1.20[a]	5.2 ± 0.80[a]
Female			
Glucose (mg/dL)	185.6 ± 48.71[a]	65 ± 10.14[b]	142.3 ± 40.52[a]
Urea (mg/dL)	51.6 ± 1.50[a]	48.6 ± 2.01[a]	45.6 ± 9.01[a]
Creatinine (mg/dL)	1.01 ± 0.10[a]	0.83 ± 0.40[a]	1.07 ± 0.40[a]
Total bilirubin (mg/dL)	0.73 ± 0.08[a]	0.95 ± 0.06[a]	0.98 ± 0.11[a]
Direct bilirubin (mg/dL)	0.46 ± 0.11[a]	0.52 ± 0.10[a]	0.57 ± 0.20[a]
AST (U/L)	90.3 ± 20.51[a]	65.2 ± 25.90[a]	81.3 ± 18.61[a]
ALT (U/L)	62 ± 12.52[a]	73 ± 20.01[a]	56.3 ± 16.52[a]
Total proteins (g/dL)	4.3 ± 1.24[a]	6.5 ± 0.40[b]	5.5 ± 0.11[a]

Values are the media ± standard error of 6 mice per group. Different superscript letters in the same row indicate significant differences ($p< 0.05$).

Ficus carica standardised in abscisic acid improves the glycemic and insulinemic responses of healthy human adults.[31]

Different mechanisms could mediate the hypoglycemic and antihyperglycemic activities of HE–Es: inhibition of intestinal glucose absorption and liver gluconeogenesis, stimulation of tissue glycolysis, stimulation of insulin release, and induction of insulin–like effects.[25,26] However, further studies must be carried out to demonstrate how the HE–Es regulate glucose levels.

Chronic toxicity evaluation

Survival, behaviour, and clinical manifestations

The chronic toxicity assay (270 days) showed that HE–Es is innocuous in BALB/c mice (LD_{50} > 1000 mg/kg b.w.). Most toxicity studies of Crassulaceae plants have been conducted in acute models (LD_{50}, mg/kg b.w.): aqueous extract of *Bryophyllum calcycinum* (1159.03 in mice and 1459.69 in rats),[10] aqueous extract of *Bryophyllum pinnatum* (660.9 in rats),[11] hydroalcoholic extract of *Kalanchoe crenata* (> 5000 in mice),[12] ethyl acetate extract of *Orostachys japonicus* (> 2000 in mice),[13] aqueous extract of *Kalanchoe gastonis* (11000 in rats),[14] and acetone extract of *Rhodiola imbricata* (> 2000 in mice).[15] In a subacute toxicity assay (14 days), the aqueous extract of *Kalanchoe integra* had an LD_{50} > 900 mg/kg b.w.[32] In a subchronic toxicity assay for 30 days, the hydroalcoholic extract of *Kalanchoe brasiliensis* showed an LD_{50} > 1000 mg/kg b.w. in mice.[33] Thus, the absence of toxicity observed in the HE–Es treated mice agrees with previous studies in other Crassulaceae species.

Mice treated with HE–Es showed normal behaviour. In this regard, all–male mice showed different grades of aggressiveness, but those treated with HE–Es were less aggressive since the first week of dose administration. The mice in the study were exposed to stressful conditions (*e.g.*, manipulation, dose administration, cage cleaning), and it has been reported that adverse environmental factors increase aggressiveness. Besides, the BALB/c mice are moderately aggressive and show intense aggressiveness episodes.[20] Moreover, the less aggressive behaviour of females has been previously described, even when they are grouped as adults.[34] The decreased aggressiveness of HE–Es treated mice could be due to the anxiolytic properties of the HE–Es; a phenomenon that has been described for other plant extracts. The ethanolic extract of *Nymphaea alba* inhibited ($p<0.01$) the aggressiveness induced by electric foot shock, decreasing the number of bites/ attacks, jumps, and confrontations.[35] The behaviour of rats treated with the aqueous extract of *Kalanchoe integra* (300 and 900 mg/kg b.w. for 14 days) was normal.[32] The aqueous extract of *Bryophyllum pinnatum* (300 mg/kg b.w.) induced analgesic effects in mice.[11] The acetone extract of *Rhodiola imbricata* (200 and 400 mg/kg b.w.) has antioxidant and hepatoprotective activity in rats, suggesting that immunomodulation contributes to such protection.[15] *Echeveria subrigida* shows adaptogenic activity since mice treated with hydroalcoholic extract are more

resistant to stress caused by anoxia, cold swimming, and forced swimming.[9] Consequently, it is suggested that the antistress effect of HE–Es could be responsible for the reduced aggressiveness of the treated mice.

In the third month of treatment, female mice of the control group showed alopecia, losing some hair on the head and whiskers; this symptom was not observed in the HE–Es treated female mice. This type of alopecia is expected in different mice strains, including BALB/c, due to excessive grooming; among the factors associated with this symptom are stress, noise, and genetic factors.[36,37] The absence of symptoms in the HE–Es treated females could be due to the antistress effect of the extract.[9]

Feed consumption, weight gain, and relative weight of organs

The reduced feed consumption after HE–Es administration in male mice could be due to flavonoids acting as IG.[7] The inhibitors delay the intestinal carbohydrate uptake and reduce postprandial glycemia, slowing gastric emptying, prolonging satiety, and reducing feed intake.[38] Furthermore, it has been reported that the IG increases the postprandial secretion of the glucagon–like peptide (GLP–1). This incretin hormone contributes to the satiety and cessation of feeding through an inhibitory effect on gastric emptying or regulating the appetite through the central nervous system.[38]

The decreased weight gain of HE–Es treated male mice could be associated with their lower feed consumption, suggesting that HE–Es regulates the appetite without inducing toxicity. Therefore, the HE–Es could be used against obesity and other eating disorders in natural formulations. This proposal is supported by the anti-obesogenic effect previously reported in studies evaluating the subchronic toxicity of aqueous extracts of *Caralluma dalziel* and *Verbesina encelioides* in mice (2000 mg/kg b.w.).[39,40]

The change in the relative weight of organs is a sensitive indicator of toxicity that could be related to effects on enzymes, physiological alterations, and target organ injuries. For example, an increased organ weight suggests hypertrophy, whereas a decreased weight suggests necrosis in the target organ.[40] In this research, the relative organ weight of female and male mice was similar (Table 1), indicating that HE–Es was non-toxic for the analysed organs. Similar results are reported in rats treated with the aqueous extract of *Bryophyllum pinnatum* (125 mg/kg b.w. for 28 days)[41] and mice treated with ethyl acetate extract of *Orostachys japonicus* (2000 mg/kg b.w.).[13]

Hematologic parameters

The hematopoietic system is sensitive to toxic substances and is affected by ingesting diverse substances. Haematological parameters provide information about the bone marrow function and intravascular effects such as hemolysis or anaemia.[42] The haemoglobin and hematocrit values of all mice were similar ($p>0.05$), suggesting that the HE–Es did not affect the erythrocytes, discarding the induction of anaemia and other related disorders. Similar results are reported for extracts of other Crassulaceae species: aqueous extract of *Bryophyllum pinnatum* in rats (165 mg/kg b.w.)[41] and hydroalcoholic extract of *Kalanchoe brasiliensis* in mice (1000 mg/kg b.w. for 30 days).[33]

The differential white blood cell count is used to evaluate the effect of substances on the immunologic system.[43] In this regard, female mice treated with HE–Es showed a decrease in the proportion of neutrophils (11.1%); however, this value is not considered pathologic since neutropenia in mice is diagnosed with values <9%.[43] Besides, this pathology was discarded because neutrophils in mice of the HE–Es satellite group increased after treatment withdrawal. On the other hand, female mice of the HE–Es group increased their number of lymphocytes. A similar effect with the number of lymphocytes and neutrophils was registered in male rats treated with an aqueous extract of *Bryophyllum pinnatum* (165 mg/kg b.w.).[41] In addition, rats treated with an aqueous extract of *Kalanchoe integra*[32] increased their levels of lymphocytes, which may be due to bacterial infection, emotional upset, or blood disorders. The lymphocytes are dynamic cells involved in the immune response; they produce antibodies against foreign microorganisms and cancer cells, among other functions.[40] The increased proportion of lymphocytes in female mice suggests a possible immunostimulant effect of HE–Es. In this regard, plant extracts have shown immunomodulatory activity. For example, the *Dendropanax morbifera* extract increased the *in vitro* proliferation of T and B lymphocytes.[44] Hydroalcoholic extracts of *Echeveria* (*i.e., E. craigiana, E. kimnachii*, and *E. subrigida*) increased the mice's splenocyte proliferation *in vitro*, highlighting the value for the *E. subrigida* extract with 34% more splenocytes.[9] This phenomenon could be associated with the increased number of lymphocytes in mice treated with the HE–Es (Table 2), supporting the immunomodulatory properties of HE–Es.

Biochemical parameters

The female and male mice of the HE–Es group showed lower glucose levels than those of control mice ($p<0.05$) (Table 3); this effect could be due to the IG activity of HE–Es. The α-glucosidase activity is essential for the intestinal absorption of carbohydrates. The glucose levels returned to normal values after the treatment was suspended, as it was registered in the HE–Es satellite group mice. Accordingly with this data, the hydroalcoholic extract of *Kalanchoe crenata* (200 mg/kg b.w.) has an antihyperglycemic effect in rats that is suggested due to an increase in insulin sensitivity.[12] Furthermore, the renal parameters urea and creatinine showed normal levels, discarding that the mice have renal failure.[42] Considering the hepatic function, the levels of total bilirubin, direct bilirubin, AST, and ALT were similar in

all mice (p>0.05), suggesting that the HE–Es did not affect the liver.[42] In general, studies of *in vivo* toxicity of Crassulaceae plants show normal liver and kidney parameters in treated animals: *Kalanchoe pinnata*,[45] *Orostachys japonicus*,[13] and *Kalanchoe brasiliensis*,[33] discarding renal or hepatic damage. On the other hand, HE–Es female treated mice showed higher levels of total proteins. This effect was also observed in rats treated with the extracts of *Rhodiola imbricata* and *Kalanchoe integra*.[15,32] In this sense, increased levels of total proteins have been associated with specific hepatic dysfunctions or animal dehydration.[46] Thus, the higher levels of total proteins in female mice of the HE–Es group may not necessarily be associated with toxic effects. It must be pointed out that the total protein values of the female mice in the HE–Es satellite group were similar to those in mice of the control group, confirming that HE–Es did not show adverse effects on the liver.

Histologic analysis of hepatic and renal tissue

Some substances can affect tissues at the cell level without inducing macroscopic observable damages; being necessary to carry out a histopathologic evaluation to identify cellular damage in organs or internal tissues.[42]

Hepatic tissue

Male mice treated with HE–Es showed macrovesicular steatosis in less than 5% of the surface area of the liver parenchyma, corresponding to zero level of steatosis in the S histologic punctuation for NAFLD.[22] This steatosis level is considered benign since the liver adequately performs its functions.[47] Besides, it must be signalled that steatosis disappeared in the male mice of the HE–Es satellite group (Figure 6). Some drugs (*e.g.*, aspirin, amiodarone, tamoxifen) also induce steatosis, which disappears once the drug is withdrawn.[48] Compared with treated male mice, females treated with HE–Es showed normal parenchyma hepatic structure without steatosis. Therefore, hormonal factors could determine the better response of females to the HE–Es treatment. In this regard, several studies showed that oestrogen signalling pathways modulate the risk of generating NAFLD. Thus, women have a lower risk of developing NAFLD up to menopause; then, women are treated with estrogens as a preventive measure. It has been reported that women with Turner disease, who lack endogenous oestrogen production, are at increased risk of developing NAFLD. Moreover, ovariectomized mice lack oestrogen production, and show a higher fatty liver phenotype that is reversed by estradiol (E2) treatment.[49]

Steatosis in male mice treated with HE–Es was only found in the zone 3; hepatocytes of this zone receive blood with less oxygen concentration and have a high concentration of NADPH and cytochrome P450; besides, this tissue functions in the detoxification and biotransformation of drugs and xenobiotics.[50] Therefore, several factors could be associated with such HE–Es associated steatosis. For example, HE–Es could be inhibiting/inactivating enzymatic systems in zone 3, increasing the exposure time to the extract, and leading to steatosis observed in the histopathologies. On the other hand, HE–Es could increase the activity of the cytochrome P450, increasing the concentration of toxic metabolites.[51]

Comparing our results with others obtained for Crassulaceae plants, the hydroalcoholic extract of *Kalanchoe brasiliensis* (1000 mg/kg b.w.) induces moderate steatosis in a subchronic toxicity assay (30 days) with male mice; the extract is classified as slightly toxic.[33] On the other hand, mice of both sexes treated with the ethyl acetate fraction of *Orostachys japonicus* (2000 mg/kg b.w., acute toxicity assay) show recruitment of polymorphonuclear leukocytes in liver tissue; the authors describe the absence of steatosis and dilation of blood vessels, associating the immune cell appearance with an overload of extract.[13]

Hepatic tissues stained with Masson's trichrome stain showed normal collagen distribution in the portal area, indicating the absence of fibrosis (Figure 7). Liver fibrosis is characterised by increased extracellular matrix (ECM) that includes different proteins such as elastin, fibrillar–type collagens (I, III, and V) and non-fibrillar (IV and VI), and glycoconjugates (*e.g.*, sulfated proteoglycans, structural glycoproteins, glycosaminoglycans such as hyaluronan). This ECM accumulation results in the destruction of the hepatic structure.[52]

Therefore, the HE–Es induced macrovesicular steatosis level zero after 270 days of administration, and could be considered slightly toxic in the liver. However, steatosis was reversible, disappearing after the suspension of the HE–Es treatment.

Renal tissue

The renal tissue slices of mice treated with HE–Es and stained with H&E showed normal structures (Figure 8), whereas those stained with Masson's trichrome showed an absence of fibrosis tubulointerstitial and glomerular, discarding glomerulonephritis (Figure 9). The kidneys are fundamental in the excretion of waste products, functioning in the filtration and reabsorption of substances.[53] The pathogenesis of chronic renal disease is characterised by the progressive deterioration of renal function and the continuous accumulation of ECM, leading to diffuse fibrosis. This process is observed as secondary glomerulosclerosis in the glomerules, whereas fibrotic characteristics are found in the tubulointerstitial space.[54] Thus, HE–Es was considered non-toxic to the kidney.

CONCLUSION

The hydroalcoholic extract of *Echeveria subrigida* showed *in vivo* hypoglycemic and antihyperglycemic activities, being innocuous in the chronic toxicity assay in mice (LD_{50} > 1000 mg/kg b.w.) and decreasing the mice's aggressiveness. Also, this extract decreased body weight and blood glucose levels, suggesting its potential as

an anti-obesity and antidiabetic agent. This manuscript describes the first study about chronic toxicity in Crassulaceae plants, particularly in *E. subrigida*. However, the antidiabetic activity and toxicity of this extract must be evaluated in other models and humans to warrant that it can be safely used in nutraceutical and pharmaceutical formulations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HE–Es: Hydroalcoholic extract of *Echeveria subrigida*; **LD_{50}:** Median lethal dose; **DM2:** Type 2 diabetes mellitus; **m.a.s.l.:** Metres above sea level; **EQ:** Equivalents of quercetin; **EI:** Equivalents of isorhamnetin; **OECD:** Organisation for economic cooperation and development; **EDTA:** Ethylenediaminetetra–acetic acid; **AST:** Aspartate aminotransferase; **ALT:** Alanine aminotransferase; **TP:** Total proteins; **TB:** Total bilirubin; **DB:** Direct bilirubin; **H&E:** Hematoxylin and Eosin Y; **NAFLD:** Non-alcoholic Fatty Liver Disease; **ANOVA:** Analysis of Variance.

SUMMARY

Flavonoids (quercetin–3-*O*-β-glucoside and isorhamnetin–3-*O*-β-glucoside) in the *Echeveria subrigida* leaves inhibit the activity of α-glucosidase *in vitro*. This study showed that the mentioned flavonoids were main components in the hydroalcoholic extract of *E. subrigida* (HE–Es). Remarkably, the HE–Es had hypoglycemic and antihyperglycemic activities *in vivo*, and was innocuous in the chronic toxicity assay.

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