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Articulo Original / Original Article

Anti-hyperglycemic activity of aqueous extracts of cells produced in *Ibervillea sonorae* (S. Watson) Green cell culture on murine model

[Actividad anti-hiperglucémica del extracto acuoso de células producidas en cultivo celular de *Ibervillea sonorae* (S. Watson) Green en un modelo murino]

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Abstract: *Ibervillea sonorae* (S. Watson) Greene, is a plant native to Mexico, where its roots have been used traditionally for treating Diabetes Mellitus. The aim of this work was to establishment of cell cultures of stem explants of *I. sonorae* and evaluation of the anti-hyperglycemic activity of cell aqueous extract on a murine model of streptozotocin-induced diabetic rats. Cell extracts had 2.29 mg palmitic acid/g extracted, and other compounds with pharmacological activities like palmitoyl ethanolamide and palmitoyl tryptamine were also identified. Diabetic rats treated with aqueous cell extract decreased glucose levels from 350 mg/dL to 145 mg/dL, AST and ALT from 164 U/L to 49 U/L and 99 U/L to 53 U/L, respectively. Additionally, there were no changes in the cellular morphology of the pancreas, liver, kidneys, and spleen. These results revealed that the cell aqueous extract from stem explants has anti-hyperglycemic activity.

Keywords: Diabetes Mellitus; Anti-hyperglycemic activity; Secondary metabolites; *Ibervillea sonorae*; Cell cultures.

Resumen: *Ibervillea sonorae* (S. Watson) Greene, es una planta originaria de México, donde sus raíces se han utilizado tradicionalmente para el tratamiento de la Diabetes Mellitus. El objetivo de este trabajo fue el establecimiento de cultivos celulares de explantes de tallo de *I. sonorae* y la evaluación de la actividad anti-hiperglucémica del extracto acuoso celular en un modelo de ratas diabéticas inducidas con estreptozotocina. El extracto celular contiene 2.29 mg de ácido palmítico/g extracto y se identificaron otros compuestos como palmitoil etanolamida y palmitoil triptamina. Las ratas diabéticas tratadas con extracto celular disminuyeron los niveles de glucosa de 350 mg/dL a 145 mg/dL, AST y ALT de 164 U/L a 49 U/L y 99 U/L a 53 U/L, respectivamente. Además, no hubo cambios en la morfología celular del páncreas, hígado, riñones y bazo. Estos resultados indican que el extracto de células de explantes de tallo de *I. sonorae* tiene actividad anti-hiperglucémica.

Palabras clave: Diabetes Mellitus; Actividad Anti-hiperglucemiante; Metabolitos secundarios; *Ibervillea sonorae*; Cultivos de células.

INTRODUCTION

Diabetes Mellitus (DM) is a chronic disease caused by insufficient insulin production by the pancreas or the inefficient use of the insulin it produces (WHO, 2018). The World Health Organization (WHO) has estimated that 422 million people worldwide have Diabetes, a figure that is likely to double in the next 20 years. Type 2 DM affects 387 million people worldwide (90% of the population with DM) and will potentially affect more than 592 million by the year 2035 (FDM, 2018), despite the availability of drugs for the treatment of DM, such as insulin, biguanides, sulfonylureas, meglitinides, thiazolidinediones, DPP-4 inhibitors, GLP-1 receptor agonists and SGLT2 inhibitors, usually accompanied by adverse effects such as hypoglycemia, weight gain, and cardiovascular complications. Therefore, the search for new efficient drugs continues, chemical synthesis being one of the alternatives. However, the yield of the synthesis is lower and less profitable due to the complexity of the metabolites. Another alternative is through *in vitro* tissue culture, where advantages such as the constant production of metabolites with anti-hyperglycemic activity can be obtained under controlled conditions. For example, in Mexico, there are at least 383 plant species used for the treatment of DM, but very few species have preclinical studies demonstrating its clinical efficacy (Andrade-Cetto & Heinrich, 2005). *Ibervillea sonorae* (S. Watson) Greene is a plant in the Curcubitaceae family used by ethnic groups in northern Mexico for the treatment of type II DM (López-Estudillo & Hinojosa-García, 1988; Jardón-Delgado *et al*., 2014) Pharmacological studies show that aqueous and dichloromethane root extracts have hypoglycemic, anti-inflammatory (Alarcon-Aguilar *et al*., 2005; Rivera-Ramírez *et al*., 2011) and anti-oxidant activity (Estrada-Zúñiga *et al*., 2012; Torres-Moreno *et al*., 2019). The production of fatty acids of callus of leaf explants *in vitro* cultures of *I. sonorae* was obtained, and its anti-oxidant activity was evaluated (Estrada-Zúñiga *et al*., 2012). Our group has tested that in the extracts of *I. sonorae* cell cultures, the flask stimulated glucose uptake is more efficacious than plant roots extract (Arciniega-Carreón *et al*., 2020).

Various studies regarding the *I. sonorae* root extract have identified the presence of metabolites with pharmacological activity such as alkaloids, coumarins, flavonoids, saponins, quinones, fatty acids (Morales & Siles, 2013; Zapata-Bustos *et al*., 2014), cucurbitacines (Achenbach *et al*., 1993; Torres-Moreno *et al*., 2015; Tripathi *et al*., 2019; Torres-Moreno *et al*., 2020), and kinoin A, B (Jardón-Delgado *et al*., 2014). The production of phenols and flavonoids has been quantified in flask cell cultures of explants stem cultured (Arciniega-Carreón *et al*., 2020). Therefore, this study aimed to establish cell cultures from explant stems of *I. sonorae* in a 0.5 L stirred tank bioreactor to obtain fatty acids, phenols, and flavonoids, as an alternative to establishing a consistent source of these kinds of metabolites; as well as the evaluation of the anti-hyperglycemic activity of the total cell extracts cultured from the bioreactor in a murine model with streptozotocin-induced Diabetes.

MATERIALS AND METHODS

Cell cultures of stem explants of I. sonorae

Ibervillea sonorae (S. Watson) Greene roots were obtained from Culiacan, Sinaloa, México, in September 2016. The identification of the plant was made by a specialist in FES Iztacala-UNAM Herbarium and voucher number assigned was 3503 IZTA. Once the stems and leaves grow, some stem explants were disinfected according to the procedure modified by Estrada-Zúñiga *et al*. (2012). were cultured in a stirred tank bioreactor of 0.5 L with a B5 medium (Gamborg *et al*., 1968), according to the methodology described by Arciniega-Carreón *et al*. (2020), 28 days, cells cultures from stem explants of *I. sonorae* were called CCSE-Is. At the end of kinetics, CCSE-Is were lyophilized (Labconco-051145636-A) at 0.0360 mbar vacuum, upper temperature: -10°C, lower temperature: -80°C and 0.0360 mbar, for 24 h for further studies.

Preparation of extracts

To identify and quantify fatty acids, 3 g of CCSE-Is lyophilizate was resuspended in 20 mL of hexane (HPLC-grade) and then sonic (2510 Branson ultrasonic bath) for 20 min; the solvent was evaporated at room temperature. This extract was called CCSE-Is-Hx.

For the analysis of total phenols and flavonoids, 3 g of CCSE-Is lyophilizate was resuspended in 30 mL of ethanol:water (8:2) and then sonicated (2510 Branson ultrasonic bath) for 20 min; later ethanol extract was centrifuged at 8000 rpm for 15 min (centrifuge: Biofuge sorvall), the solvent was evaporated at 50°C under vacuum in a rotary evaporator (BÜCHI Heating Bath B-490), the extract was called CCSE-Is-EtOH.

To evaluate the anti-hyperglycemic activity on a murine model, the CCSE-Is lyophilizate (50

mg/kg) was resuspended and sonicated in 300 µL water for 20 min extract was called CCSE-Is-Aq.

Quantification of fatty acids of CCSE-Is-Hx

The quantification of fatty acids from CCSE-Is-Hx was performed by fluorometry with 9-8 diethylamino-5H-benzo [α] phenoxazine-5-one (Nile red) according to Chen *et al*. (2009), with a cell concentration between 1 x 10^4 - 1x10^6 cells/mL. The stained sample was immediately placed in a plastic fluorometer cell (Jenway 6280) and excited at 540 nm to take the fluorescence emission measured at 475 nm. To calculate the mg concentration of fatty acids, a Triolein type curve (Sigma Aldrich®) was performed. These determinations were made on day seven of culture suspension.

Extraction and quantification of phenols and flavonoids of CCSE-Is-EtOH

The quantification of total phenols of CCSE-Is-EtOH was performed with the modified method of Singleton *et al*. (1999). The concentration of total phenols was measured by spectrophotometry, based on a colorimetric oxidation-reduction Folin-Ciocalteu reagent. Absorbance was measured in a spectrophotometer (Perkin Elmer Lambda XLS) at 760 nm. The calibration curve used gallic acid as standard, and the results were expressed in mg of gallic acid per g of extract in dry weight (mg GAE/g of extract).

The quantification of total flavonoids of CCSE-Is-EtOH was made based on the methodology described by Gracia Nava (2007). Absorbance was measured in a spectrophotometer (Perkin Elmer Lambda XLS) at 510 nm. The calibration curve used Rutin as standard, and the results were expressed in mg of Rutin per g of extract in dry weight (mg Rutin/g of extract).

Analysis of metabolite families of CCSE-Is-EtOH by TLC

Analysis of metabolites of CCSE-Is-EtOH was carried out by thin-layer chromatography (TLC). The phytochemical tests in TLC consisted of identifying sterols using the Salkowski reaction, identification of saponins with the Lieberman-Burchard test, and phenolic acids identified with the Folin-Ciocalteu reagent.

Analysis of fatty acid of CCSE-Is-Hx by HPLC-PAD

Analysis of High-Performance Liquid Chromatography-Photodiode Array Detection was performed with 3 g of CCSE-Is-Hx. The recovered extract was weighed, and the yield was calculated, expressing it as mg of extract per g of biomass. HPLC-PAD was performed in Waters alliance 2695 equipment, which was coupled to Waters 2998 diode array detector that was programmed to take readings in a wavelength range of 200 to 400 nm. As the mobile phase, a combination was used under isocratic conditions using methanol and 1% acetonitrile (HPLC grade Merk) at a ratio (10:90), the flow rate in the range of 0.8 mL/min. All samples were analyzed by duplicate injecting 10 µL standards (Palmitic acid, Stearic acid, Pentadecanoic acid, and Lauric acid) and 10 µL of CCSE-Is-Hx.

Analysis of metabolites of CCSE-Is-EtOH by UPLC-MS

For UHPLC-Q-TOF–MS, an Agilent 1290 Infinity II system coupled with a 6545 Q-TOF–MS of dual ESI source (Agilent Technologies, Santa Clara, CA, USA) was utilized. The mobile phase was Milli-Q water containing 0.1% formic acid (component A) and acetonitrile with 0.1% formic acid (component B). For the organic phase analysis, an Agilent Eclipse XDB-C-8. A 4.6 mm, 5 µm column running at 0.3 mL/min was used as standard LC-MS to separate extracts obtained with gradient elution from 40 to 60% B; 15–20 min, isocratic 90%; and 20-27 min, isocratic 10% B. The mobile phase flow rate was 0.3 mL/min after injecting 10 µL. The column temperature throughout the separation process was 40°C. Previously to perform the LC-MS analyses, samples were kept at 0.5°C. The ESI source was operated in a positive ion mode with the following conditions: The fragment voltage was set at 100 V, the nebulizer gas was set at 40 psi, the capillary voltage was set at 3500 V, the drying gas flow rate was set at 10 dm^3/min, and the temperature was set at 300°C. For MS/MS measurements, a collision energy ramp ranging from 15 to 40 eV to promote fragmentation was used. The data was acquired in centroid and profile modes using the high-resolution mode (4 GHz). The mass range was set at 50-1000 m/z in the MS and MS/MS modes. The data was processed with the Mass Hunter Workstation LC-MS Data Acquisition B.08.00 Software.

Evaluation of the anti-hyperglycemic activity of CCSE-Is-Aq

Adult male Wistar rats, 8 weeks old and weighing 250 ± 50 g, were purchased from FES Iztacala-

UNAM Bioterium. They were kept in standard conditions at 22-23°C and with light-dark periods of 12 h/12 h. The animals were fed with standard diet (LabDiet, 5012) and water. All animal experiments were handled according to Official Mexican Standards (NOM-033-ZOO-1995; NOM-062-ZOO-1999). The Laboratory Animal Ethics Committee approved this research of the National School of Medicine and Homeopathy of the National Polytechnic Institute (ENMH-CB-145-2015), which complies with Internationals Regulations and Policies.

Hyperglycemia was induced in rats by a single intraperitoneal (i.p.) injection of 50 mg/kg streptozotocin (STZ, Sigma, Aldrich). On day 7 after injection, blood glucose was measured with a glucometer (Accucheck Active Roche®). Those animals with blood glucose >150 mg/dL were considered diabetic. To evaluate the anti-hyperglycemic effect, CCSE-Is-Aq was administered orally using a gastric tube. Animals were classified into five groups, each with 7 rats, as follows: Group I was the healthy control; Group II was the diabetic control; Group III was the vehicle-treated diabetic group (H_20); Group IV was the diabetic group treated with Metformin (100 mg/kg), and group V was the diabetic group treated with CCSE-Is-Aq (50 mg/kg). Ten doses were administered every three days. After the treatment period, the animals were sacrificed according to Norma Oficial Mexicana, (1995). Blood samples for biochemical tests and small samples of the pancreas, liver, kidney, and spleen were also obtained for histopathology studies.

Measurement of weight and peripheral glucose levels

The weight was monitored weekly on an analytical balance, the data obtained was registered in individual files and documented for subsequent statistical analysis. Peripheral glucose levels were obtained with a glucometer from the distal tail portion of the animals in all the treatments throughout the experimental period (Accu-check active®).

Hematic biometry

A blood sample containing 20 µL of heparin was obtained to analyze hemoglobin, leukocytes, erythrocytes, platelets, and hematocrit. It was processed on an Electronic Hemogram instrument through the electronic impedance technique.

Biochemical parameters

Blood was obtained through cardiac puncture. This fluid was collected without anticoagulant and then centrifuged at 570×g for 10 min at 4°C and processed on a Cobas Mira Roche instrument. Biochemical parameters included serum glucose levels, hepatic enzymes such as ASpartate amine Transferase (AST) and ALanine amine Transferase (ALT), lipid profile including cholesterol, triglycerides, high-density lipoprotein (HDL), and low-density lipoprotein (LDL), and renal function based on the urea and creatinine levels.

Histological studies

Animals were euthanized, quickly dissected, and small samples of the pancreas, liver, kidney, spleen, and stomach were dipped and stored in formaldehyde fixative solution in PBS 10%. Slices 4–6 µm in size were cut for histological studies. The slices were stained with hematoxylin and eosin (H&E) for examination under microscopy (100X and 400X) to observe possible damage to the architecture of the tissue.

Statistical analysis

The experimental data for antidiabetic activity was analyzed using analysis of variance with a *post-hoc* Bonferroni's multiple comparison test. **p* values ≤0.05 were considered statistically significant. We used GraphPad Prism ver. 6.0 (GraphPad Software, Inc.).

RESULTS

Fatty acids quantification in CCSE-Is

The maximum production of Fatty acids of CCSE-Is was 609.76 ± 2.33 mg Triolein/g at 21 days of culture.

Phenols and flavonoids in the CCSE-Is-EtOH

We performed quantifications during the growth kinetics from 2.43 ± 0.01 GAE/g of cells in the bioreactor at 21 days of culture. The production of flavonoids in the cell cultures was from 1.26 ± 0.01 mg Rutin/g of cells in the bioreactor at 21 days of culture.

Metabolites families in CCSE-Is-EtOH

Table No. 1 shows the retention factor (Rf) of the metabolites family identified in the Lieberman-Burchard reaction. This reaction indicates the presence of steroidal saponins. The Folin-Ciocalteu reagent had shown the presence and capacity of

phenolic acids to react with oxidizing agents. The Salkowski reagent showed the presence of sterols and methyl sterols in the CCSE-Is-EtOH.

Table No. 1
Rf values of the families of metabolites identified in CCSE-Is-EtOH*

Family	Rf
Saponins	0.5
Phenolic acids	0.05
Sterols	0.25

CCSE-Is-EtOH: Ethanolic extract of cell cultures of stem explants of *I. sonorae

Fatty acid in CCSE-Is-Hx by HPLC-PAD

Figure No. 1 shows palmitic acid (A) and the presence of palmitic acid of 2.29 mg/g of CCSE-Is-Hx (B) extract.

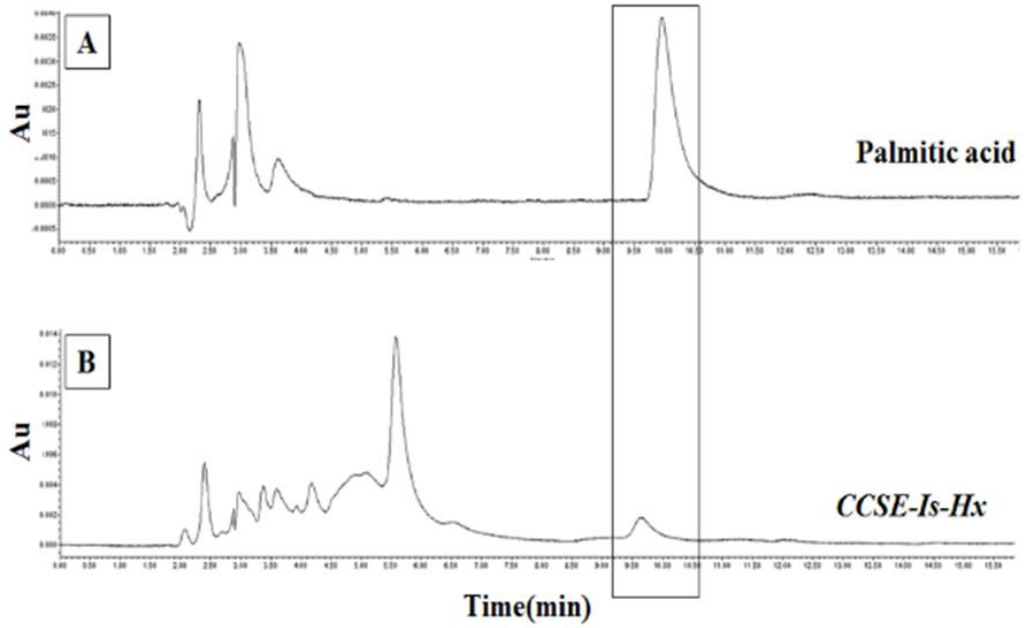

Figure No.1
HPLC chromatograms of palmitic acid and CCSE-Is-Hx. A) Standard calibration curve of palmitic acid. B) CCSE-Is-Hx. Stationary phase: C18; Mobile Phase: Methanol: 1% acetonitrile (10:90); λ: 220 nm; Flow rate: 0.8 mL/min, column and sample temperature: 25°C. *CCSE-Is-Hx: Hexanic extract of cell cultures of stem explants of *I. sonorae.*

Metabolites analysis in CCSE-Is-EtOH by UPLC-MS

Approximately 40 peaks were detected. Chromatographic peaks were tentatively identified by comparing the MS data with databases based on the search of m/z values of molecular ion peaks in the positive mode $[M+H]^+$. Five principal phytochemical compounds were identified: palmitoyl ethanolamide, palmitoyl tryptamine, hippeastrine, citepressine I, and claisarinol (Table No. 2).

Table No. 2
Proposed phytochemical constituents in CCSE-Is-EtOH* using UPLC-MS

Rt (min)	[M + H]+ (m/z)	Proposed compound	Formule	Score	Theoretical mass
7.509	300.2898	Palmitoyl Ethanolamide	C18 H37 N O2	84.46	299.2825
6.882	421.3178	Nb-Palmitoyltryptamine	C26 H42 N2 O	90.17	398.3286
5.413	316.1182	Hippeastrine	C17 H17 N O5	98.6	315.1109
7.013	415.2113	Clausarinol	C24 H30 O6	98.06	414.204
5.305	302.1022	Citpressine I	C16 H15 N O5	85.55	301.0948

CCSE-Is-EtOH: Ethanolic extract of cell cultures of stem explants of *I. sonorae

Anti-hyperglycemic activity of CCSE-Is-Aq

To demonstrate the anti-hyperglycemic activity of CCSE-Is-Aq, we used an *in vivo* model of streptozotocin-induced diabetic rats. We observed that initial glucose levels in diabetic groups were higher than 300 mg/dL and the glucose in the healthy group of 100 mg/dL. We found that the group of diabetic rats treated with CCSE-Is-Aq showed a reduction of the peripheral glucose levels from 350.80 ± 36.20 mg/dL to 145.30 ± 18.00 mg/dL. These values compared to the glucose values of the group treated with Metformin at 100 mg/kg, that shown a decrease from 264.20 ± 20.60 mg/dL to 127.40 ± 10.30 mg/dL (Figure No. 2). These glucose values of the group treated with CCSE-Is-Aq are not statistically different from the healthy group, only with the diabetic group.

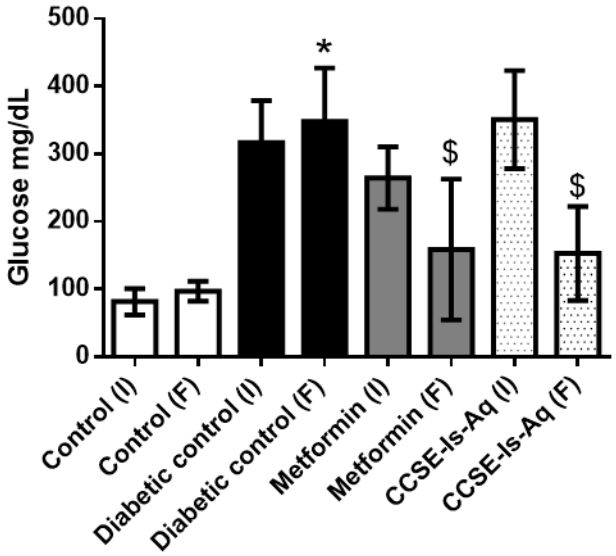

Figure No.2
Peripheral glucose levels at initial treatment (I) and after 10 doses (F). Heatlhy control: Untreated animals; Diabetic control: Untreated diabetic rats, Metformin: Diabetic rats treated with metformin (100 mg/kg) and CCSE-Is-Aq: Diabetic rats treated with aqueous extract of cell culture of *I. sonorae* (50 mg/kg). Values are expressed as mean ± SEM (n=7). One-way analysis of variance (ANOVA) followed by a Bonferroni *post*-hoc. $p<0.05$ was considered as significant. *Statistically significant difference versus the Healthy control group. $ Statistically significant difference versus the diabetic control group

Weight of rats

Table No. 3 illustrates the weight gains of the experimental groups after day 14 and 21 of treatment with treatment administration every third day. We observed that on day 14, the group of diabetic rats treated with CCSE-Is-Aq had an average weight gain of 88.38 ± 19.40 g, these values did not show statistically significant differences with respect to the healthy group, which gained 62.88 ± 3.87 g. However, we did find a significant statistical difference with respect to the diabetic group on day 14 with a weight gain of 32.00 ± 13.14 g. At day 21, the weight gain was 84.00 ± 4.42 g in the healthy group and in the group treated with CCSE-Is-Aq it was surprisingly 100.10 ± 21.26 g.

Table No. 3
Weight after 10 doses of treatment with CCSE-Is-Aq* in streptozotocin-induced diabetic rats

Group	Weight gain day 14 (g)	Weight gain day 21 (g)
Healthy control	**62.88 ± 3.87**	**84.00 ± 4.42**
Diabetic control	**32.00 ± 13.14**	**45.63 ± 16.14**
Metformin	**48.75 ± 6.59**	**63.25 ± 9.49**
CCSE-IS-Aq	**88.38 ± 19.40***	**100.10 ± 21.26**

Heatlhy control: Untreated animals; Diabetic control: Untreated diabetic rats, Metformin: Diabetic rats treated with metformin (100 mg/kg) and CCSE-Is-Aq: Diabetic rats treated with aqueous extract of cell culture of *I. sonorae* (50 mg/kg). Values are expressed as mean ± SEM (n=7). One-way analysis of variance (ANOVA) followed by a Bonferroni *post*-hoc. $p<0.05$ was considered as significant. *Statistically significant difference versus the control group. *CCSE-Is-Aq: Aqueous extract of cell cultures of stem explants of *I. sonorae*

Hematic Biometry

The values obtained in the blood biometry analysis as shown in Table No. 4, the results of measured parameters in rats, resulted in blood cells: red blood cells, white blood cells, and platelets as within reference levels; therefore, it is inferred to rats treated with CCSE-Is-Aq does not affect levels of white and red bloodlines in our experiment.

Biochemical Parameters

Table No. 5 shows the biochemical profile for the rats group that were experimented on with and without treatment. The central serum glucose of the group treated with CCSE-Is-Aq shows levels 132.70 ± 6.04 mg/dL after 10 doses. There are no statistical differences compared to the healthy group with 118.3 ± 16.42 mg/dL. The diabetic group shows levels of 364.30 ± 7.35 mg/dL at 21 days. These levels have statistical differences compared to the group treated with CCSE-Is-Aq. In the hepatic profile, the AST and ALT enzyme levels of the group treated with CCSE-Is-Aq were not statistically different from the healthy. Moreover, these results have statistical differences with the diabetic group. The group treated with CCSE-Is-Aq compared with the reference values showed no statistical differences concerning the healthy group in the blood lipid and renal profile.

Histological studies

In the histopathological analysis of different experimental groups, Figure No. 3 shows normal architecture in the pancreas of the experimental group treated with CCSE-Is-Aq, similar to the healthy group. Islets of Langerhans were observed with a similar structure to the healthy group and a greater number of cells in the central part of islets of Langerhans. Likewise, the pancreatic acini and centroacinar cells presented a normal architecture. Unlike the diabetic group, where the architecture of the damaged tissue was, the islets of Langerhans had an elongated morphology with widely dispersed beta cells.

Table No. 4
Hematic biometry of different experimental rats groups

Formula	Parameter	Reference	Healthy	Diabetic	Metformine	CCSE-Is-Aq*
White Formula	Platelets (x 103/µL)	428-857	786±125	345±599	495±231	494±154
	Leukocytes (x 103/µL)	4.84-12.96	6.36±4.91	8.53±1.47	9.70±3.25	12.23±2.18
	Eosinophils (x 103/µL)	0.1	1.66±1.20	1	0.66±0.33	1.33±0.66
	Basophils (x 103/µL)	0	0.33±0.33	0.66±0.33	0	1±0.57
	Monocytes (x 103/µL)	0-2	4.5±4.5	1.66±0.88	1.66±0.33	1±0.57
	Lymphocytes (x 103/µL)	82.67-96.71	67.67±11.35	71.67±6	57±4.35	47.67±2.84
Red Formula	Erythrocytes (x 106/µL)	6.33-8.64	8.68 ±516	8.47 ±421	7.70±613	7.92 ±224
	Hemoglobin (g/dL)	12.09-15.18	14.53±0.71	14.13±0.40	14.57±1.04	14.70±0.52
	Hematocrit (%)	33.1-45.8	47.53±3.12	48.03±3.30	44.53±3.39	44.27±1.46
	Medium Corpuscular Volume (fL)	52.4-59.9	54.47±0.033	54.40±2.19	56.80±1.20	55.83±0.74
	Mean Hemoglobin Concentration (pg)	15.6-21.2	16.63±0.26	16.07±0.21	18.87±0.14*$	18.53±0.33*$
	Mean Corpuscular Hemoglobin Concentration (g/dL)	28.1-93.3	30.57±0.46	29.60±1.20	32.70±0.15	33.20±0.20$

Heatlhy control: Untreated animals; Diabetic control: Untreated diabetic rats, Metformin: Diabetic rats treated with metformin (100 mg/kg) and CCSE-Is-Aq: Diabetic rats treated with aqueous extract of cell culture of *I. sonorae* (50 mg/kg). Values are expressed as mean ± SEM (n=3). One-way analysis of variance (ANOVA) followed by a Bonferroni *post*-hoc. $p<0.05$ was considered as significant. *Statistically significant difference versus the Healthy control group. $ Statistically significant difference versus the diabetic control group. *CCSE-Is-Aq: Aqueous extract of cell cultures of stem explants of *I. sonorae*

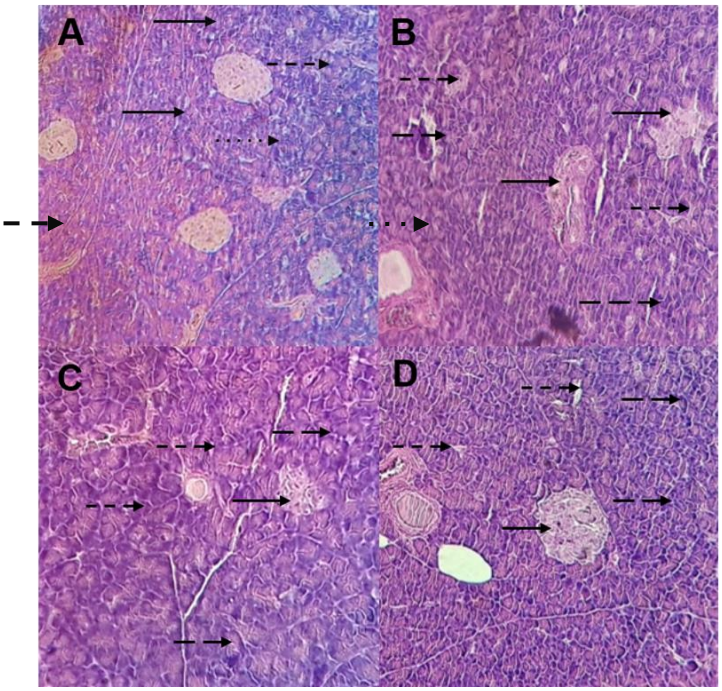

Figure No. 3
Histology of the pancreas of the experimental groups. A) Healthy control, B) Diabetic control, C) Diabetic rats treated with Metformin, D) CCSE-Is-Aq*. Islets of Langerhans( ), pancreatic acini ( ) and centroacinar cells ( ). Observed at 100X. * CCSE-Is-Aq: Aqueous extract of cell cultures of stem explants of *I. sonorae*

Table No. 5
Blood chemistry analysis of different experimental rat groups

Profile	Parameter	Reference	Healthy control	Diabetic control	Metformin	CCSE-Is-Aq*
	Central Glucose (mg/dL)	58-160.2	118.3±16.42	364.3±7.35	155.3±13.17$	132.7±6.04$
Hepatic Profile	Aspartate amine transferase (AST) (U/L)	63-175	126.7±26.19	164±19.70	159.7±13.54	48.67±24.14$ +
	Alanine amine transferase (ALT) (U/L)	17-50	66.00±15.62	99.33±15.17	68.67±5.81	52.50±12.12
Lipidic profile	Total cholesterol (mg/dL)	33-50	50.33±12.81	46.33±2.84	51±4.04	43.33±0.88
	High density lipoproteins (HDL) (mg/dL)	-	17±4.35	14.33±2.40	15.33±2.40	17.17±1.32
	Low density lipoproteins (LDL) (mg/dL)	-	22±6.80	20±3.51	23.67±2.90	24.17±2.16
	Very low-density lipoproteins (VLDL) (mg/dL)	-	11.33±1.76	12±1.15	12±1	11.17±1.28
	Triglycerides (mg/dL)	55-115	58.67±9.38	63±7.76	60.33±4.25	59.50±6.58
Renal profile	Urea (mg/dL)	68.5-111.8	57.33±9.82	53±9.07	53±9.07	52.50±3.93
	Creatinine (mg/dL)	0.71-2.29	1.20±0.15	0.96±0.24	1.16±0.21	0.96±0.03

Heatlhy control: Untreated animals; Diabetic control: Untreated diabetic rats, Metformin: Diabetic rats treated with metformin (100 mg/kg) and CCSE-Is-Aq: Diabetic rats treated with aqueous extract of cell culture of *I. sonorae* (50 mg/kg). Values are expressed as mean ± SEM (n=7). One-way analysis of variance (ANOVA) followed by a Bonferroni *post*-hoc. $p<0.05$ was considered as significant. $ Statistically significant difference versus the diabetic control group. + Statistically significant difference versus the Metformin group. *CCSE-Is-Aq: Aqueous extract of cell cultures of stem explants of *I. sonorae*

Figure No. 4 shows the liver of the different experimental groups, healthy, Metformin and CCSE-Is-Aq. We observed normal polygonal-shaped hepatocytes and normal hexagonal-shaped liver lobules with central vein sinusoidal capillaries. Hepatic lesions such as degeneration of hepatocytes or intracellular accumulations or processes of inflammation, fibrosis, necrosis were not observed. In the diabetic group, an altered morphological architecture was observed with a greater presence of sinusoidal spaces and rupture of the central vein.

Figure No. 5 shows kidney sections of the healthy group, and animals treated with CCSE-Is-Aq show a normal renal architecture of Hassal corpuscles and proximal and distal tubules. The diabetic group without treatment showed tubules of different sizes.

Figure No. 6 shows histological sections of the spleen of animals treated with CCSE-Is-Aq, which showed maintained structural integrity in the healthy group: integrity of the capsule and a balanced distribution of white splenic pulp and red splenic pulp integrity were observed. The stromal architecture and structure observed showed no damages, also valid for the healthy group structures. The diabetic group had an altered morphology where a clear distinction between white and red pulps was not observed.

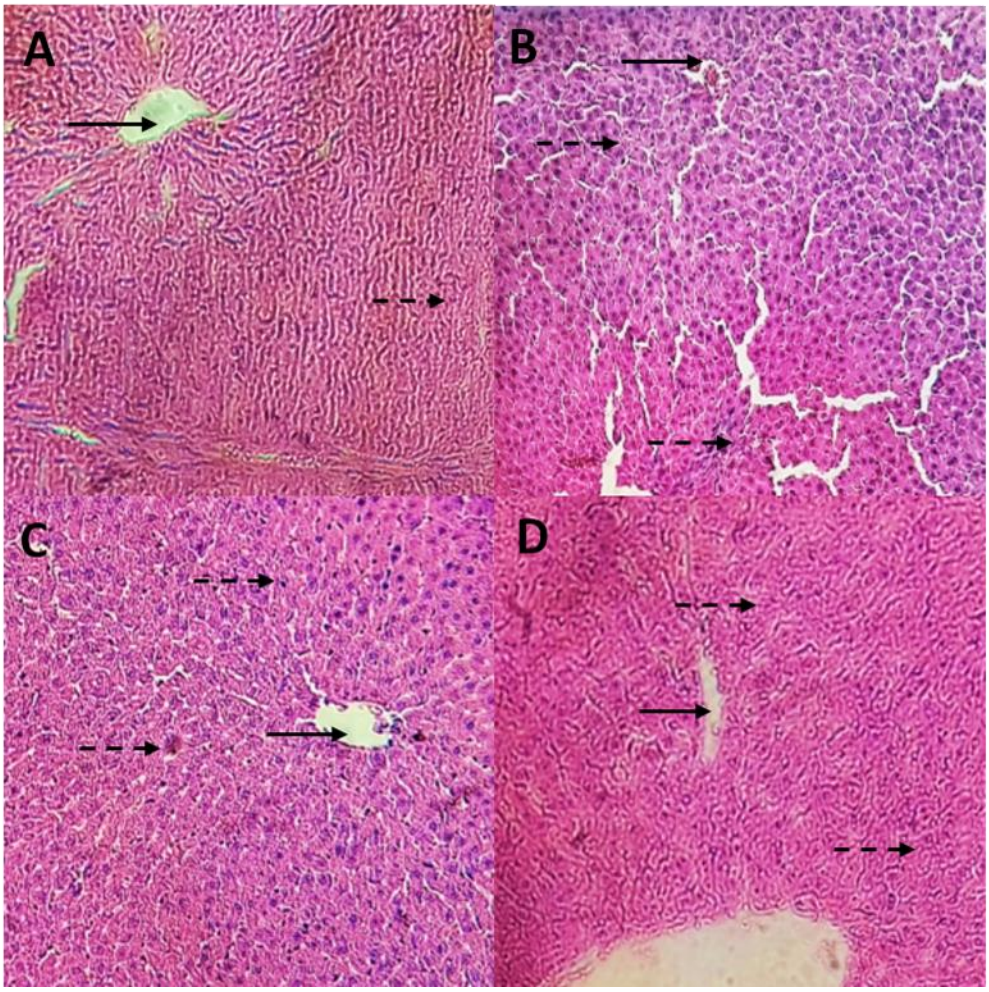

Figure No. 4
Histology of liver of different experimental groups. A) Healthy control, B) Diabetic control, C) Diabetic rats treated with Metformin, d) CCSE-Is-Aq*. Central vein (⟶) and sinusoidal capillaries (⇢). Observed a 400X. * CCSE-Is-Aq: Aqueous extract of cell cultures of stem explants of *I. sonorae*

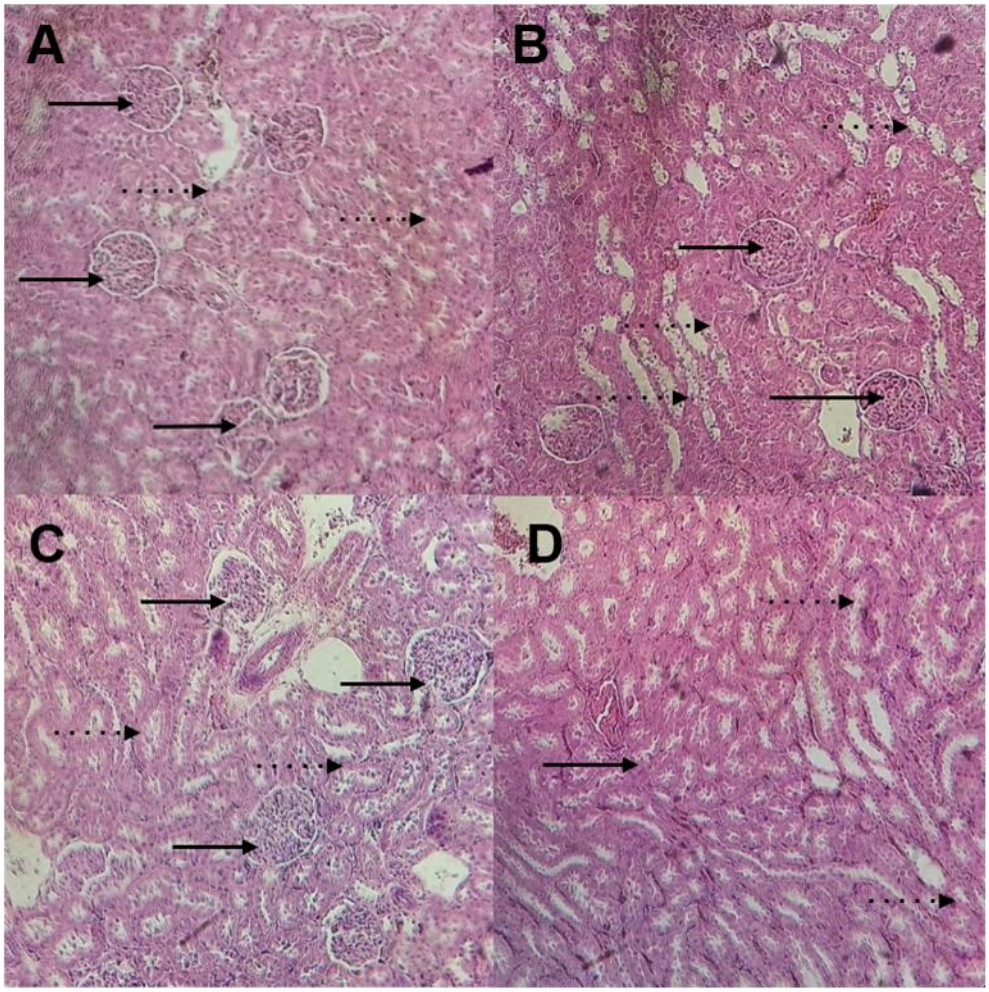

Figure No. 5
Kidney histology of experimental groups. A) Healthy control, B) Diabetic control, C) Diabetic rats treated with Metformin, D) Diabetic rats treated with CCSE-Is-Aq*. Hassal corpuscles (⟶) and Proximal-distal tubules (- -▸). Observed at 400X. * CCSE-Is-Aq: Aqueous extract of cell cultures of stem explants of *I. sonorae*

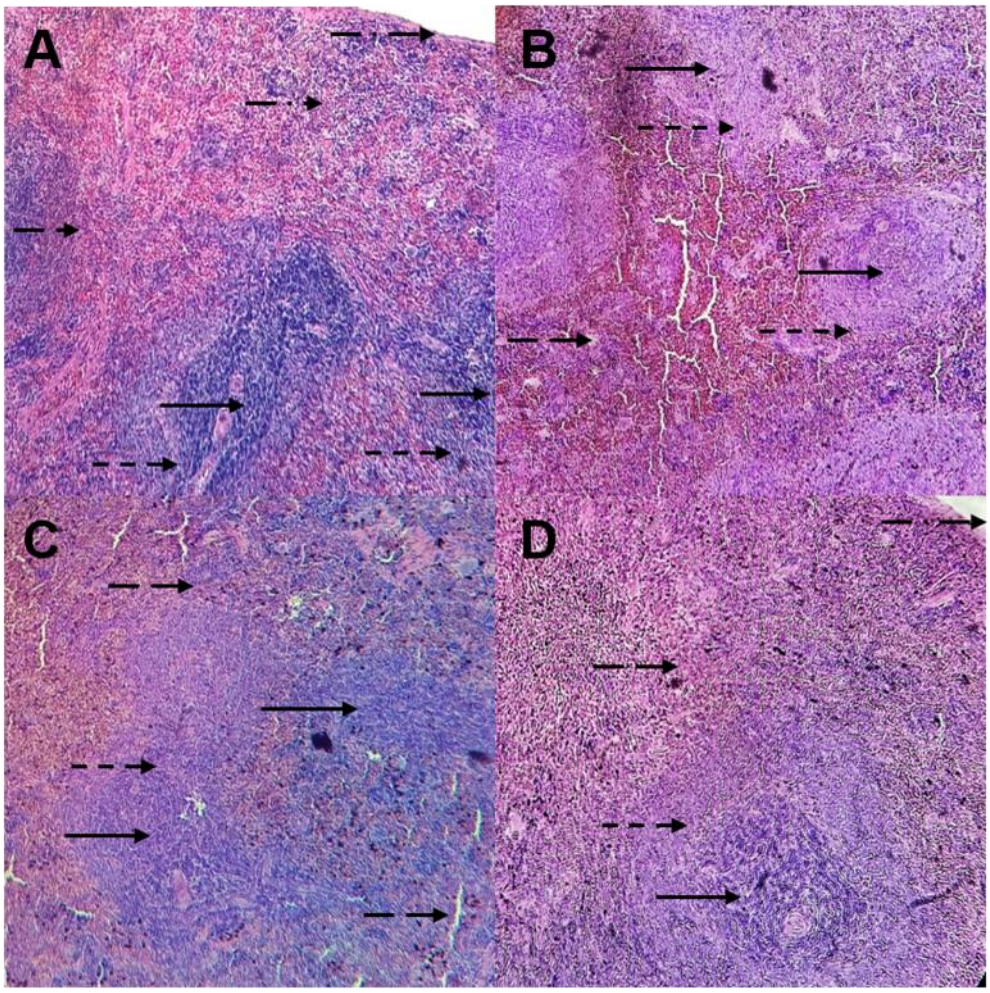

Figure No. 6
Histology of spleen of experimental groups. A) Healthy control, B) Diabetic control, C) Diabetic rats treated with Metformin, D) Diabetic rats treated with CCSE-Is-Aq*. White splenic pulp (⟶), red splenic pulp (— →) and stromata (— · →) and Capsule (– →). Observed at 100X. *CCSE-Is-Aq: Aqueous extract of cell cultures of stem explants of *I. sonorae*

DISCUSSION

Studies are being carried out to explore the possibility for an alternative supply route through biotechnological production of biomass/product using shoot cultures in a bioreactor *(Tripathi et al*., 2019). To face these challenges, industries and scientists are looking for possibilities of alternative resources to produce plant pharmaceuticals utilizing plant cell cultures. In recent years, the advances in biotechnology, particularly methods for culturing plant cells, have provided good strategies for the commercial processing of plant cell cultures, thus becoming a viable alternative to produce these compounds of pharmacological interest. Initially, we evaluated the production of fatty acids, and during the growing interval, the data showed higher values of fatty acids in CCSE-Is with 609.76 ± 2.33 mg Triolein/g. Other studies have reported fatty acids in the callus of leaves with 48.57 mg/g (Estrada-Zúñiga *et al*., 2012) and those obtained from the root with 0.25 mg/g (Hernández-Galicia *et al*., 2007). These differences in results are attributed to the plant explants, the kinetic duration of cell culture, and the culture times in which we carry out the quantifications. We also observed that the production of fatty acids is associated with cell growth during the kinetics of cell culture in the bioreactor.

Furthermore, we evaluated the production of

phenols in CCSE-Is-EtOH, and at this point, we obtained 2.43 ± 0.01 mg GAE/g of extract at 21 days of culture. We also obtained a total flavonoid content of 1.26 ± 0.01 mg Rutin/g of extract at 21 days of culture. These values of flavonoids are similar to those obtained by Arciniega-Carreón *et al*. (2020), from *I. sonorae* suspended cell cultures in a flask (from stem explant), in which they obtained 1.10 ± 0.03 mg Rutin/g. In other studies, the concentration of flavonoids was higher than that obtained with other Cucurbitaceae in callus extracts from *Momordia charantia* L. where 2.90 mg/g of flavonoids biomass (kaempferol, quercetin, and luteolin) were produced at 42 days of culture (Ahmed *et al*., 2015), such as α-tocopherol (42.93 µg/g FW), and total folate (0.72 µg/g FW) (Saini & Keum, 2017). Interestingly, the production of phenols and flavonoids declined after day 21. The decrease of these metabolites in bioreactor culture (data not shown) was probably influenced by oxidizing agents, lack of nutrients, or enhanced accumulation of stress metabolites (Ali *et al*., 2013). The transfer of cell suspension cultures in shake-flasks to bioreactors represents a critical step towards producing secondary metabolites without cells losing the biosynthetic potential for producing biomass and secondary metabolites (Godoy-Hernández *et al*., 2000). The production of phenols and flavonoids in bioreactor cell culture was probably altered due to the effect of shear stress and the physicochemical properties of the cultures, along with the physical and mechanical properties of the bioreactors, having different consequences concerning the loss of viability, lower cell growth rate (Raposo & Lima-Costa, 2006) or an increase in the synthesis of secondary metabolites (Henzler, 2000; Trejo-Tapia *et al*., 2005; Trejo-Tapia *et al*.,2007).

Regarding the identification of the metabolites in CCSE-Is-Hx, we quantified 2.29 mg/g extract of palmitic acid. In previous studies, fatty acids like lauric acid, myristic acid, penteadecanoic acid, stearic acid, palmitic acid, and monoglycerides were isolated from *I. sonorae* roots extract dichlorometane (Hernández-Galicia *et al*., 2007). Estrada-Zúñiga *et al*. (2012) identified lauric acid, myristic acid, penteadecanoic acid, stearic acid, palmitic acid from *I. sonorae* callus cultures (from leaf explants). Also, in other metabolite families, cucurbitane-type glycosides kinoin A, B and D in extracts of roots were identified (Achenbach *et al*., 1993; Kamalakkannan & Prince, 2006; Jardón-Delgado *et al*., 2014). In UPLC-MS analysis of CCSE-Is-EtOH, we identified different metabolites such as palmitoyl pthanolamide, a member of the fatty-acid ethanolamide family (Hoareau & Roche, 2010; Paterniti *et al*., 2015; Pesce *et al*., 2020), palmitoyl tryptamine alkaloid derived from tryptamine (Lúcio *et al*., 2015; Kumar *et al*., 2018), hippeastrine, an alkaloid which has been identified in amaryllidaceae family (Gasca *et al*., 2020; Martinez-Peinado *et al*., 2020), citepressine I, acridone alkaloid identified in the Rutaceae family (Bissim *et al*., 2019; Ye *et al*., 2021) and the phenolic compound claisarinol, which has been identified in the Anonnaceae family (Rangsinth *et al*., 2019). This is the first time that these metabolites are reported in CCSE-Is-Aq at the Cucurbitacea family.

Using chromatographic column separation and NMR spectroscopy, cucurbitacin IIb was isolated and identified as a chemical component of the crude methanolic extract from this plant (Torres-Moreno *et al.*, 2020). Amin *et al*. (2019), identified palmitic acid in seeds of the native and hybrid variety of *Cucurbita maxima* Linn with a total of 22.78 mg/kg (native) and 2.84 mg/kg (hybrid), respectively. HPLC has identified other metabolite families as phenolic acids and flavonoids such as gallic acid 15.65 mg/mL (Zapata-Bustos *et al*., 2014), tannic acid, catechin, epicatechin, caffeic acid, p-coumaric acid, gentisic acid, and chlorogenic acid (Sur & Ray, 2020). These are the first studies of metabolites of CCSE-Is. Based on the reported studies of *I. sonorae* root extracts, we infer that the phenolic compounds, and fatty acids present in CCSE-Is influence their anti-hyperglycemic activity.

On the other hand, the current research offers new findings with anti-hyperglycemic activity. In this regard, there are previous studies about the anti-hyperglycemic effect of roots extracts of *I. sonorae* in normoglycemic and diabetic mice and rats. Administration of 300 mg/kg of aqueous root extract of *I. sonorae* in healthy mice decreased peripheral glucose from 41.40 ± 1.50 to 36.50 ± 2.30 mg/dL at 360 min (Alarcon-Aguilar *et al*., 2002), and 600 mg/kg dichloromethane root extract diminished glycemic levels from 400.70 ± 18.40 to 95.60 ± 23.60 mg/dL glucose at 240 min in alloxan-induced diabetic mice (Alarcon-Aguilar *et al.*, 2005). When dichloromethane root extract was evaluated at 300 mg/kg, it decreased from 374.00 ± 9.50 to 63.00 ± 7.30 mg/dL glucose at 240 min (Hernández-Galicia *et al*., 2007). In this study, the evaluation of anti-hyperglycemic activity in streptozotocin-induced diabetic rats treated with CCSE-Is-Aq showed

decreased peripheral glucose levels from 350.80 ± 36.20 mg/dL to 145.30 ± 18.00 mg/dL after 10 doses of 50 mg of CCSE-Is-Aq/kg. Studies with aqueous root extract have shown the inhibition of α-glucosidase and stimulated insulin secretion from RIN-m5F pancreatic β cells (Banderas-Dorantes *et al*., 2012). In other studies, a potential increase of blood insulin levels in Wistar rats when treated with extract of roots of *I. sonorae* showed an increase in insulin secretion of RIN-m5f β cells, suggesting this could also exert its antidiabetic effects by an insulin-secretagogue activity on the pancreatic β cells (Sur & Ray, 2020). Nevertheless, it was demonstrated that Cucurbitaceae species had demonstrated their hypoglycemic activity and their everyday use for the treatment of Diabetes, and this activity is attributed to the production of some families of metabolites such as flavonoids, terpenes, sterols, and steroids (Andrade-Cetto & Heinrich, 2005). However, the mechanism of action of the metabolites of this plant is unknown, and there is no technique for the consistent and controlled production of these compounds.

During treatment, an increase in weight in the rat was observed in the healthy and CCSE-Is-Aq groups. These results suggest that the administration of CCSE-Is-Aq does not present any adverse effect. In streptozotocin-induced diabetic rats, increased food consumption and decreased body weight were observed due to excessive break-down of tissue proteins, as reported by Kamalakkannan & Prince, (2006). This effect could be due to better control of the hyperglycemic condition in diabetic rats. Decreased blood glucose levels could improve body weight in streptozotocin-diabetic rats (García-Galicia *et al*., 2014), and therefore these results suggest a favorable improvement in body weight.

A decrease or increase in white and red blood cells means an alteration in the immunology of the organism, reflected in symptoms such as anemia, leukemia, or thrombocytopenia. In this study, we observed as evidence of improvement, that the levels of hemoglobin, platelets, leucocytes, and erythrocytes (Table No. 4) in the hematic biometry of the diabetic group treated of CCSE-Is-Aq was significantly improved, similar to the healthy group (p<0.05) and compared to the untreated diabetic rats. Therefore, we can infer that there were no immunological alterations in the groups treated with CCSE-Is-Aq.

The blood chemistry analysis (Table No. 5) exhibited serum central glucose levels in normal levels of 132.70 ± 6.04 mg/dL were no statistical difference compared to the healthy group of 118.39 ± 16.42 mg/dL, with statistical differences compared to the diabetic group of 364.30 ± 7.35 mg/dL. The studies reported on extracts from the root of *I. sonorae* suggest that this hypoglycemic effect may have been due to improved insulin sensitivity and/or reduced insulin resistance (Rivera-Ramírez *et al.*, 2011).

Regarding enzyme levels (Table No. 5) such as AST and ALT in the hepatic profile, the diabetic rats group treated with CCSE-Is-Aq showed similar values to the healthy group (p<0.05). Similar observations were reported for other plant extracts, showing a significant recovery in the parameters of streptozotocin-induced diabetic rats (Al-Faris *et al*., 2010). These enzymes determine hepatic and myocardial functionality; its increase indicate necrotic processes, but its decline results from clinical improvement. Therefore, the CCSE-Is-Aq extract does not cause damage to the liver tissue. In the study of the concentrations of different types of fats in the blood lipid profile, levels of total serum cholesterol, VLDL cholesterol, LDL cholesterol, triglycerides levels, and decreased level of HDL cholesterol of the group treated with CCSE-Is-Aq, do not present significant differences against the healthy control. This was observed in other studies with medicinal plants, such as *Euryale ferox*, *Glycosmis pentaphylla*, *Tridax procumbens* and *Mangifera indica* which demonstrate glycosylation of proteins, oxidative stress, chronic hyperglycemia, and cardiovascular diseases through the altered lipid profile (Petchi *et al*., 2014; Ahmed *et al*., 2015). As Diabetes is a metabolic disorder, it is characterized not only by increased glucose concentrations but also by the altered lipid profile level (Ahmed *et al*., 2015). Therefore, the plausible mechanism of action of CCSE-Is-Aq in controlling the blood glucose level might be the enhancement of secretion insulin from pancreatic β-cells. Furthermore, the lipid profile mechanism might be due to the improved glycogenesis in the liver. The renal function profile of the group treated with CCSE-Is-Aq showed levels within the reported parameters, similar to the healthy group (p<0.05). Diabetic nephropathy and hyperglycemia are the leading cause of end-stage kidney disease, and high levels of urea and creatine are indicators of this kidney failure (Wang *et al*., 2013). The streptozotocin-induced diabetes model has been widely used to study diabetic kidney changes (Neuhofer & Pittrow, 2006). In brief, the CCSE-Is-Aq could be beneficial for the treatment of

diabetic nephropathy.

Histopathological studies related to the pancreas, liver, kidney, and spleen sections revealed the disturbed morphological features (Figure No. 3, Figure No. 4, Figure No. 5 and Figure No. 6) in the diabetic group. Islets of Langerhans contained β-cells to nearly normal in streptozotocin-induced diabetic treated mice after 10 doses of CCSE-Is-Aq similar to the healthy group. Damaged central vein and hepatocytes returned to normal in the liver of streptozotocin-induced diabetic mice treated with 10 doses of CCSE-Is-Aq compared to the diabetic control. Kidney spleen tissues in the group treated with CCSE-Is-Aq showed a normal architecture like the healthy group, and the diabetic group had damage in tissue structure. Compared to that of the normal control group, the architecture of these tissues in the diabetic group was consistent with other studies (Punithavathi *et al*., 2011; Sedigheh *et al*., 2011). It is evident from the results in the group treated with CCSE-Is-Aq compared to the person with Diabetes is favorable for the repair and restoration of pancreas, liver, kidney, and spleen tissue and had a protective function that helped maintain the architecture of the tissues. This hepatoprotective mechanism is probably due to unsaturated fatty acids, such as oleic acid and linoleic acid, in pumpkin seed, reducing cholesterol levels in rats (Takada *et al*., 1994).

Also, our results revealed that the extract did not modify the architecture of the tissues of the group treated with our extract of cell cultures on a 0.5 L stirred tank bioreactor compared to the healthy group. Hyperglycemia can cause the glycoxidation of different molecules, leading to advanced glucose end products (AGEs). This applies to both albumin and specific lipoproteins. These AGEs have pro-inflammatory and pro-oxidant effects, firstly by stimulating the secretion of inflammatory cytokines; secondly by activating the production of reactive oxygen species (ROS). Hence the importance of metabolites with anti-oxidant activity such as those identified in our extract. citepressine I and polyphenols such as claisarinol can also reduce the oxidative stress generated by reactive oxygen species (ROS) (Bissim *et al*., 2019; Rangsinth *et al*., 2019; Ye *et al*., 2021). Therefore, palmitoyl ethanolamide has been studied due to its analgesic, antiallergic and anti-inflammatory activities. The anti-inflammatory properties of palmitoyl ethanolamide arise from its ability to antagonize the nuclear factor κB (NF-κB) signaling pathway through the selective activation of the PPARα receptors (Hoareau & Roche, 2010; Paterniti *et al*., 2015; Pesce *et al*., 2020). Palmitoyl ethanolamide constitutes an attractive therapeutic tool for Diabetes and has demonstrated efficacy and great promise of its use in treating inflammatory disorders in an animal model.

For the above mentioned, we propose a new alternative to produce metabolites with anti-hyperglycemic activity and avoid the indiscriminate use of this species. The method could improve the study of the mechanisms of action and elucidate the metabolites of *I. sonorae*.

CONCLUSION

Our results show that CCSE-Is maintain fatty acids, phenolic compounds, flavonoids, and CCSE-Is-Aq that has been grown in the bioreactor (50 mg/kg) in streptozotocin-induced diabetic rats have anti-hyperglycemic activity. In addition, it controlled weight loss, enzyme levels of AST and ALT decreased, and the cellular morphology of the organs was not affected. Even the islets of Langerhans were repaired and did not show toxicity to the liver, kidney, and spleen. This is the first time that the anti-hyperglycemic effect of CCSE-Is-Aq grown in a bioreactor has been evaluated in an animal model of Diabetes. The results confirm the traditional use of *I. sonorae* for the treatment against Type 2 DM.

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