

Effects of *Cissampelos sympodialis* Extract in a Murine Model of Streptozotocin-Induced Diabetes

Ana Tereza Cerqueira Lima¹, Laise Cedraz Pinto¹, Tatiane Oliveira Teixeira¹, Samara dos Santos Suzarth¹, Keina Maciele Campos Dourado¹, Karina Carla de Paula Medeiros², Márcia Regina Piuvezam³, Lain Carlos Pontes de Carvalho⁴, Neuza Maria Alcantara-Neves¹ and Camila A Figueiredo^{1*}

¹Institute of Health Sciences, Federal University of Bahia, Campus Canela, Salvador, Bahia, Brazil

²Department Morfologia- CB, Federal University of Rio Grande do Norte, Natal, Rio Grande do Norte, Brazil

³Immunopharmacology Laboratory, Department of Physiology and Pathology, UFPB, João Pessoa, Paraíba, Brazil

⁴Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, Salvador, Bahia, Brazil

Abstract

Cissampelos sympodialis (Cs) is an herb used for the treatment of several diseases due to its anti-inflammatory properties. The present study investigated the effect of oral administration of Cs extract on STZ-induced diabetes in mice. Diabetes was induced by three i.p. injections of STZ (100 mg/Kg) in male BALB/c mice. The mice were randomly divided into four groups: control group, STZ group, Cs 0.4 group (0.4mg/Kg) and Cs 4 group (4mg/Kg). At the 50th day of protocol mice were euthanized and serum insulin and fasting blood glucose were determined by ELISA and electrical feeder, respectively. Changes in pancreas architecture were assessed through histopathological examination using hematoxylin-eosin method. We also evaluated cytokine (IL-10, IL-4, and IL-17) production in spleen cell culture from diabetic mice stimulated or not with pokeweed mitogen by ELISA. The treatment with Cs extract was not able to reduce blood glucose levels or to increase insulin production in any of the time points evaluated after STZ injection. However, treatment with Cs extract was able to prevent damage on islets' morphology. Additionally, Cs extract at the

concentrations evaluated was not able to modulate cytokine production in vitro by spleen cells. *Cissampelos sympodialis* can indirectly contribute to the reduction of the inflammatory response characteristic on STZ-induced diabetes in mice, which might be associated to the reduction of the damage on the islets.

***Corresponding Author:** Camila Figueiredo, Department of bioregulation, Health Sciences Institute of the Federal University of Bahia, Brazil; E-mail: cavfigueiredo@gmail.com

Introduction

Type 1 Diabetes Mellitus (DM1) affects children, adolescents and young adults and which prevalence is rising during the last decade [1]. The World Health Organization (2013) estimates that diabetes will be the 7th leading cause of death in 2030.

The clinical manifestations of this metabolic disorder can be observed when about 80% of the β -cells have been destroyed during the disease pathogenesis [2] which is characterized as an autoimmune process where neutrophils, macrophages, dendritic

cells, lymphocytes and pro-inflammatory cytokines such as IFN-gamma and IL-17 may play a role [3].

Exogenous insulin therapy is the main therapeutic approach in patients with type 1 Diabetes Mellitus (DMI) [4]. However, while insulin therapy slows down the disease, it does not prevent chronic disease complications. Moreover, treatment of DMI has always posed a challenge to balance hyperglycemia control with hypoglycemia episodes [5, 6].

New immune therapies strategies have been studied to treat diabetes mainly because insulin and also oral hypoglycaemic drugs have such undesirable side effects as the hypoglycemia episodes [7]. In this context, *C. sympodialis* (Cs) is commonly used by Brazilian folk medicine mainly in Southeast and Northeast Brazil, to treat inflammatory disorders and our group has already demonstrated its pharmacological potential on animal asthma models [8-11] and also it has been described in a case report to treat autoimmune Psoriasis [12]. Therefore, the present study aimed to investigate the influence of oral administration of Cs extract on glycemia, insulin and histopathological changes in the pancreas as well as the

cytokine profile on STZ-induced Type I Diabetes Mellitus (DMI) in mice.

Methods

Plant material and extract preparation

Leaves of *Cissampelos sympodialis* Eichl. (Menispermaceae) were obtained from the Botanical Garden of the Laboratório de Tecnologia Farmacêutica/Universidade Federal da Paraíba (voucher specimen Agra 1456). The standardized Cs extract was obtained according to Cerqueira-Lima, Alcântara-Neves, Pontes- de-Carvalho, Costa, Barbosa-Filho et al. [10].

Experimental model of autoimmune diabetes

The 50-day experimental protocol is shown in [Figure 1]. The treatment regiment was adapted from previous works in the literature [13-15]. Also, the experimental model of Type I Diabetes (DMI) was induced with 100 mg/Kg of streptozotocin (STZ) adapted from Deeds' Anderson, Armstrong, Gastineau, Hiddinga et al. [16].

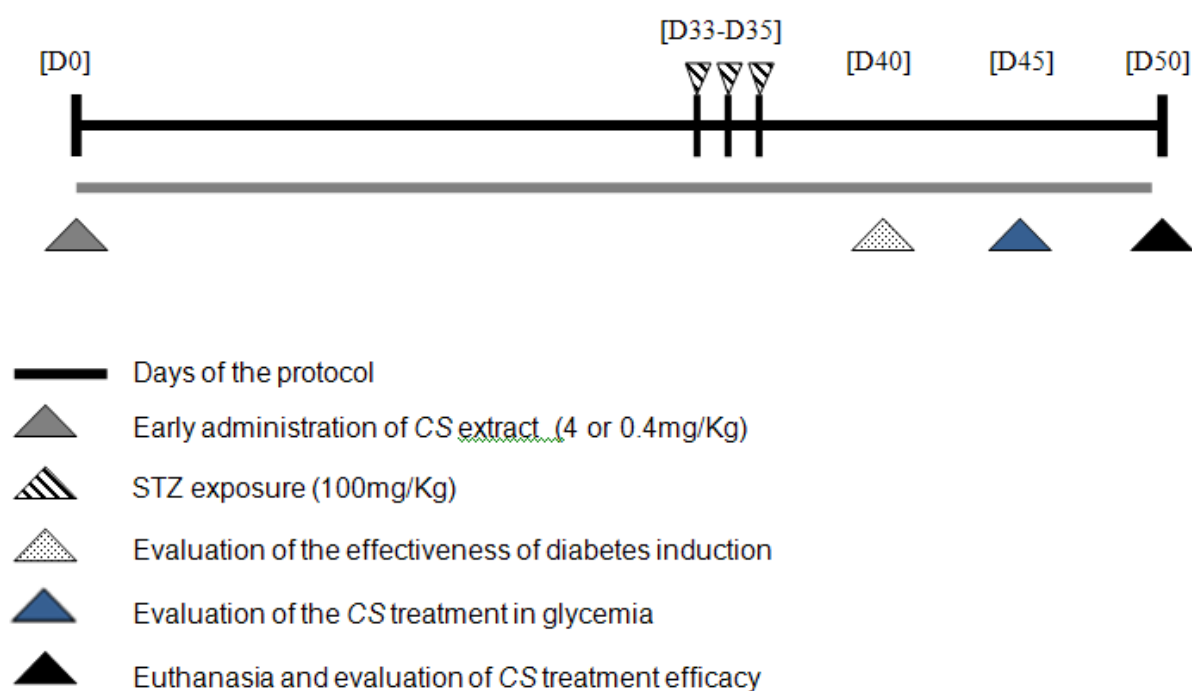


Figure 1: Experimental design of STZ-induced autoimmune diabetes

BALB/c mice (7-8 weeks) were divided into four groups: 1) control group (received saline orally and three consecutive intraperitoneal (i.p.) injections of citrate buffer pH 4.5); 2) STZ group (received saline orally and three i.p. injections of STZ-100 mg/Kg); 3) Cs 0,4 group (received 0,4mg/Kg Cs extract orally and three i.p. injections of STZ-100 mg/Kg) and 4) Cs 4 group (received 4mg/Kg Cs extract orally and three i.p. injections of STZ-100 mg/Kg). After 5 days of STZ injection, the effectiveness of diabetes induction was evaluated by measuring blood glucose (>200mg/dL). The animals were housed at 22°C and received water and food ad libitum during the experiment. STZ injections were applied to animals with 8 hours fasting. Fasting was also applied previously to the measurement of blood glucose. This protocol was previously approved by the Ethics Committee of the Institute of Sciences of Health- Federal University of Bahia, CEUA/ICS 029/2012.

Evaluated parameters

Fasting glycemia was assessed on day [D0], [D40], [D45] and [D50] and was determined from blood obtained from the tail vein by electrical feeder (Accu-Chek Active, Roche).

Insulin was measured by ELISA (Ultrasensitive Mouse Insulin ELISA, Mercodia, Uppsala, Sweden) on serum from controls and diabetic mice.

For the histological analysis the samples were embedded in paraffin twice. Using a microtome, the paraffin blocks were cut into 3- μ m seriate sections. In the staining phase, the slides were immersed in hematoxylin-eosin. In the dehydration phase, the structures went through three containers with absolute alcohol and two containers with xylol. Histopathological alterations was done by two different pathologists in a blind-fashion coordinated by Dr. Karina CP Medeiros from Department of Morphology, Rio Grande do Norte Federal University, Natal, Rio Grande do Norte, Brazil.

Reading was performed with light microscopy (Nikon Labophot) at 100X. The pancreatic islet area was measured with the aid of Image G software. Furthermore, the atrophy of the islets was scored (0: normal, 1: mild, 2: moderate, 3: severe) by

a pathologist and overall injury of pancreatic islets was further calculated according to the sum of the score. We selected 15 randomly areas at High-Power Field (HPF, 100x) of each blind sample.

Spleen cells from diabetic animals, after euthanasia, were distributed into the culture plates in the presence of the Cs extract, in dilutions of 50, 100 and 200 μ g/ml, and in the presence of Pokeweed (PWM) mitogen. Control group cells were cultured in the absence of the extract but also were stimulated with PWM. After incubation (37°C, 5%, CO₂, 24h), the cell culture supernatants were collected and quantification of IL-10, IL-4, IL-17 was done by ELISA (BD Pharmingen®, USA).

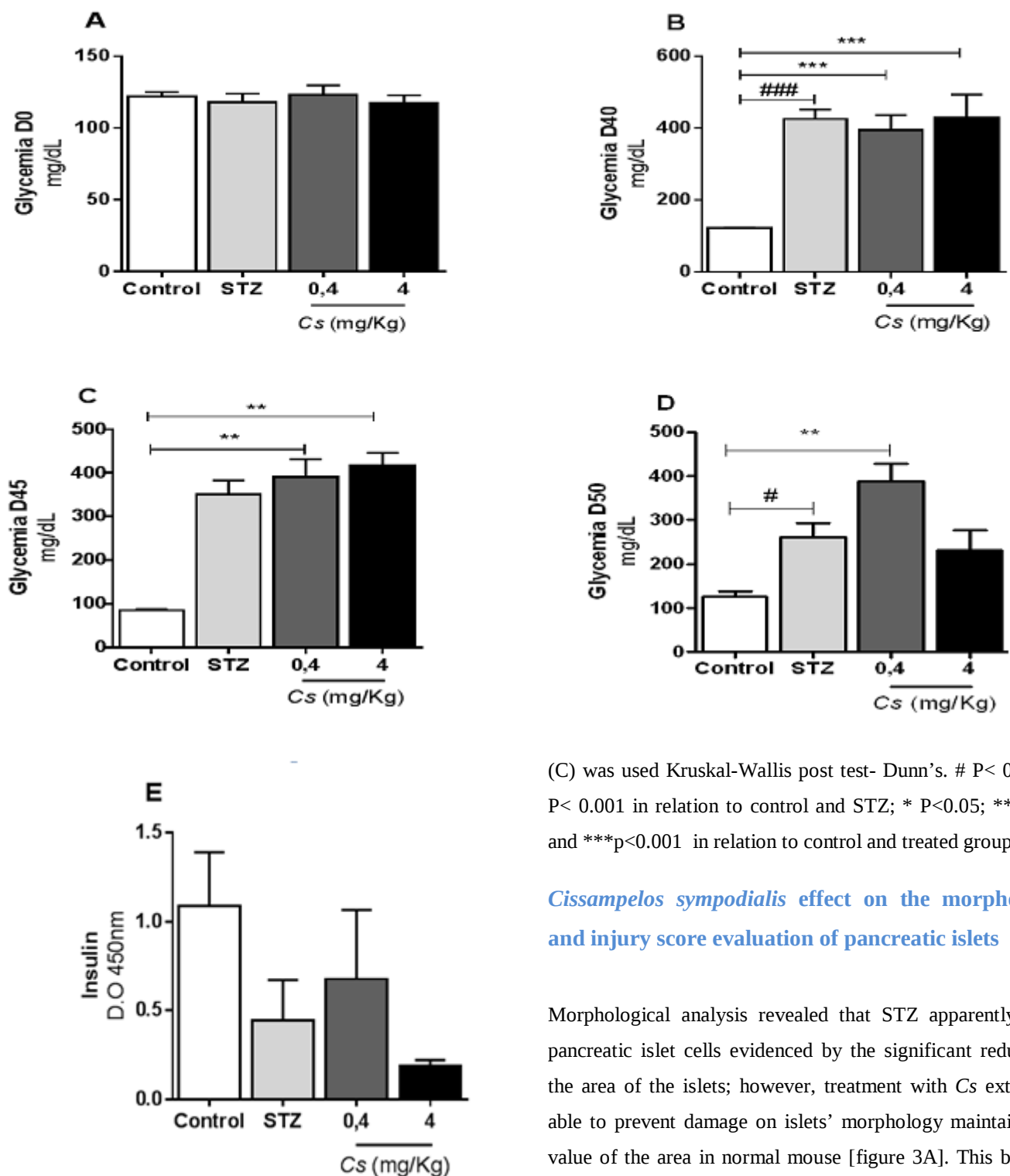
Statistical analysis

The One-way (ANOVA) analysis of variance and Tukey or Green House-Geisser correction as post-test (for data with normal distribution) and Kruskal- Wallis and Dunn's as post test (for data without normal distribution) were used to determine statistical significance between groups on GraphPad v6 (GraphPad Software Inc., San Diego, CA, USA). The results were considered significant when $P < 0.05$.

Results

Cissampelos sympodialis effect on the production of glycemia and insulin

As expected injections of STZ increased the amount of glucose and reduced insulin production in animals. STZ is a natural nitrosourea glycoside isolated from *Streptomyces achromogenes*. The injection of STZ is capable of forming neoantigens generating an inflammatory response by mimicking the immune mechanism [17]. As seen in [Figure 2 B, C and D] treatment with Cs extract was not able to reduce blood glucose levels in any of the time points after injection with STZ. The same was observed in insulin production [Figure 2E]. [Figure 2A] presents the baseline glycemia. Although not statistically significant, Cs extract at the lower dose (0,4mg/Kg) was able to partially modulate insulin production [Figure 2E].



(C) was used Kruskal-Wallis post test- Dunn's. # $P < 0.05$; ### $P < 0.001$ in relation to control and STZ; * $P < 0.05$; ** $p < 0.001$ and *** $p < 0.001$ in relation to control and treated groups.

Cissampelos sympodialis effect on the morphometric and injury score evaluation of pancreatic islets

Morphological analysis revealed that STZ apparently shrank pancreatic islet cells evidenced by the significant reduction in the area of the islets; however, treatment with Cs extract was able to prevent damage on islets' morphology maintaining the value of the area in normal mouse [figure 3A]. This beneficial use of Cs extract was further confirmed by analysis of score where one can observe the improvement of pancreatic injury [figure 3B].

Figure 2: Effect of oral treatment with hydro alcoholic extract of *Cissampelos sympodialis* (CsE), in mice experimentally exposed to STZ in glycaemia and insulin production. The graphics (A, B, D and E) was used ANOVA-Tukey's. Graphic

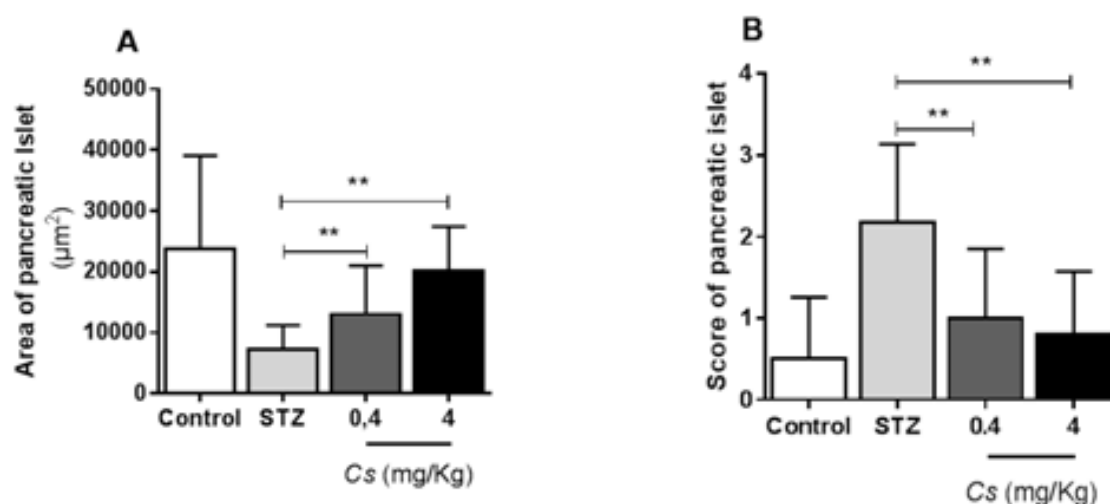


Figure 3: Morphometric analysis of pancreatic islet (A) and pancreas pathological scores in different groups (B). CS: *Cissampelos sympodialis*. Data appear as mean $\pm$ SD *** $P < 0.000$ and ** $P < 0.001$ in relation to STZ and treated groups.

Cissampelos sympodialis effect on the histopathological changes in pancreas

The normal mouse showed pancreatic architecture preserved with large pancreatic islets between the various serous acini [figure 4A]. STZ caused shrinkage of islets of Langerhans in diabetic mice [figure 4B]. The significantly higher number of active islets of Langerhans in the Cs extracts treatment indicated that Cs possibly either protects the β -cells against death and/or assisted the regeneration of partially destroyed β -cells [figure 4C and D].

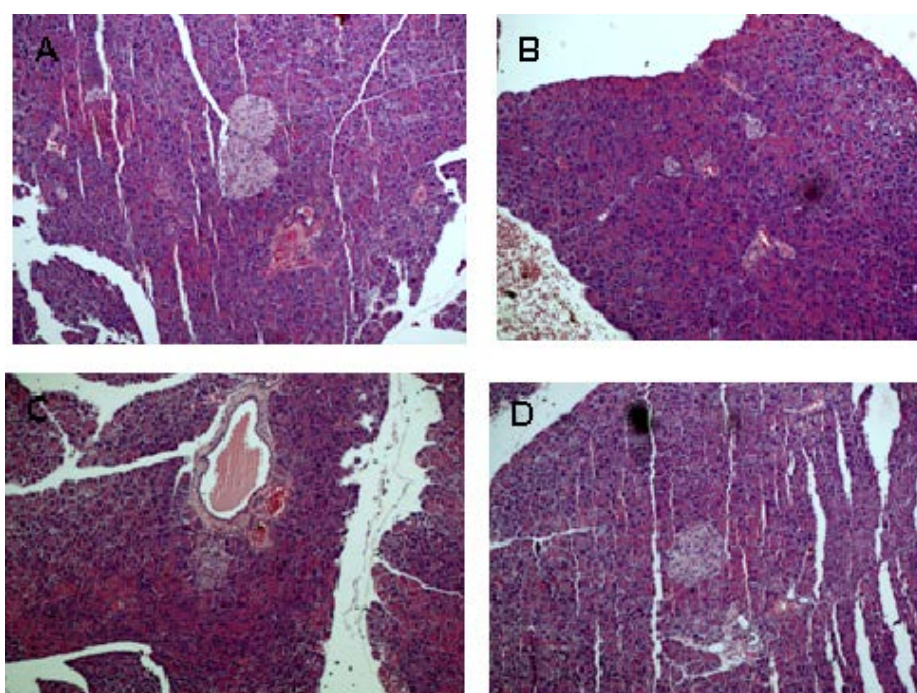


Figure 4: Representative comparisons of pancreatic tissues from: (A) pancreas of normal mouse (H&E, $\times 100$); (B) pancreas of diabetic mouse (H&E, $\times 100$); (C) pancreas of CS treatment diabetic mouse at a dose of 0,4 mg/kg (H&E, $\times 100$); and (D) Pancreas of CsE treatment diabetic mouse at a dose of 4 mg/kg (H&E, $\times 100$).

diabetic mouse at a dose of 0,4 mg/kg (H&E, $\times 100$) and (D) Pancreas of CsE treatment diabetic mouse at a dose of 4 mg/kg (H&E, $\times 100$).

Cissampelos sympodialis effect on the cytokine production in vitro

Spleen cells from mice subjected to in vivo protocol was put into plate in the presence or absence of Cs extract. The results suggest, even with no statistical difference, a tendency to reduction of the cytokine IL-17 [Figure 5C] important for

maintaining the inflammatory response in an experimental model of diabetes induced by STZ. There was also a trend to increased IL-10 [Figure 5A] and IL-4 [Figure 5B] cytokines that can help with their respectively immune regulatory properties and counter balancing the Th1/Th17 inflammation.

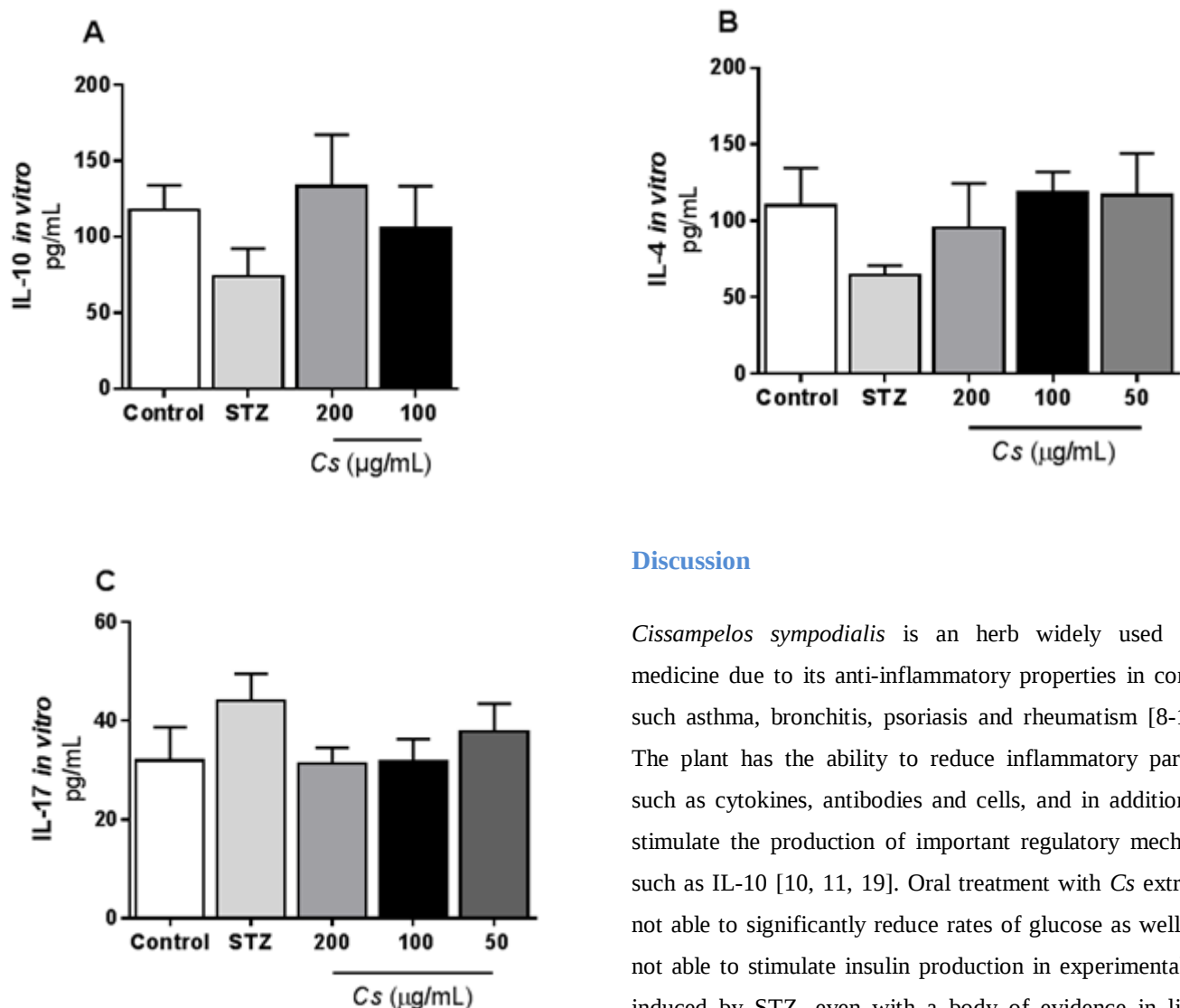


Figure 5: *Cissampelos sympodialis* effect on cytokine production in vitro. The graphic (A and B) was used ANOVA-Tukey's. Graphic (C) was used ANOVA-Green House-Geisser correction. It was not possible to detect any IL-10 concentration (A) when splenocytes were exposed to the Cs concentration of 50µg/ml.

Discussion

Cissampelos sympodialis is an herb widely used in folk medicine due to its anti-inflammatory properties in conditions such asthma, bronchitis, psoriasis and rheumatism [8-12, 18]. The plant has the ability to reduce inflammatory parameters such as cytokines, antibodies and cells, and in addition it can stimulate the production of important regulatory mechanisms, such as IL-10 [10, 11, 19]. Oral treatment with Cs extract was not able to significantly reduce rates of glucose as well as was not able to stimulate insulin production in experimental model induced by STZ, even with a body of evidence in literature describing possible mechanisms which could prevent inflammation and consequently reduce the inflammatory process characteristic of the pathophysiology in type I diabetes [10-12, 18]. Spelman, Burns, Nichols, Winters, Otters berg, 2006; Alexandre-Moreira, Freire-de-Lima, Trindade, Castro-Faria-Neto, Piuvezam, [19]. Semwal, Semwal, Vermaak, Viljoen [20] published a review on the

therapeutic properties of *Cissampelos*, the antidiabetic property was cited. The fact that we found no reduction in glycemia our model can in part be explained by the work done by Colomeu, Figueiredo, Cazarin, Schumacher, Maróstica [21] which suggest that reduction of glycemia on diabetic animals when treated with natural products may occur at 17th week of treatment only however we were not able to extend our protocol for that.

DMI is believed to be initiated by physiological β - cell death or islet, sources of insulin, injury triggering the homing of macrophages and dendritic cells that in turn launch an inflammatory reaction. The infiltrating macrophages secrete pro-inflammatory cytokines, as well as various chemokines that attract immune cells such as dendritic cells, macrophages and T lymphocytes. T cells recognising β -cell specific antigens become activated infiltrate the inflamed islets and attack the β -cells [22].

In our study, histopathological examination and injury score of the islets of pancreas showed that Cs extract-treated animals had a better preservation of pancreatic islets compared to STZ animals. In this context despite being a type 1 diabetes autoimmune disease our data suggest that treatment with Cs extract can reduce the damage islets or stimulus the development of new β -cells [24]. Veld [24] showed in a review that patients with chronic type 1 diabetes show evidence of sustained β -cells apoptosis possibly suggesting that in patients with type 1 chronic diabetes there is a continuous formation of new β -cells. The better preservation of islets when the animals were treated with Cs extract can be directly related to the anti-inflammatory property of Cs [10, 12, 18, 19, 24]. Previous phytochemical works have described the presence of five tertiary alkaloids in the hydro alcoholic Cs extract: warifteine, metilwarifteine, roraimine, liriodenine and milonine [25, 26]. Warifteine (Wa) is the more abundant alkaloid in Cs, having its anti-inflammatory and immune modulatory properties mainly related to the modulation of the production and migration in vivo and in vitro of inflammatory cells such as neutrophils, eosinophils, macrophages and lymphocytes [10, 18, 27].

Cytokine production was evaluated in vitro in our work. Although not statistically significant, it was possible to observe that there was a tendency in modulating IL-17 in animals treated with Cs extract. IL-17 is important in diabetogenesis as well as IFN- γ [3]. The trend to increased IL-4 and especially IL-10 is an important parameter to support the hypothesis that Cs extract has the ability to control inflammation in our model. Spelman, Burns, Nichols, Winters, Ottersberg [24] showed in a review that *Cissampelos sympodialis* is a plant capable of producing both cytokines, IL-4 and IL-10. Although not observed in this study, there is no doubt, based on other studies that *Cissampelos sympodialis* has the ability of up-regulate IL-10 production [10, 11, 19, 24]. The immune modulatory action of IL-10 results on the inhibition of proinflammatory cytokines, such as TNF- α , decreasing the activity of neutrophils, macrophages, T lymphocytes and reducing the expression of Major Histocompatibility Complex (MHC) proteins class II [9-11, 19, 24], where all these parameters play a role on diabetes installation.

Conclusion

In this study, we showed that *Cissampelos sympodialis* is able to indirectly contribute to the reduction of the inflammatory response characteristic of this disease. The reduction of the damage on the islets may be connected to the reduction of the inflammatory response. Treatment with Cs extract can help reduce the damage caused by the islet on STZ-inflammatory process.

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