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## *Prosopis strombulifera* aqueous extract reduces T cell response and ameliorates type I diabetes in NOD mice



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### ABSTRACT

**Background:** New products with tolerogenic properties on T cell response are necessary to improve current efficacy, cost and side effects of immunosuppressants. *Prosopis strombulifera* aqueous extract (PsAE) have reported cytotoxic, antitumoral, antiatherogenic and antileishmanial activities, containing phytochemicals with immune-related activities. Despite these, there are no previous studies with respect to PsAE suppressive properties over T cell proliferation and their function.

**Goal:** Because of previous antecedents, this study aims to evaluate the effect of PsAE on T cell activation, proliferation, cytokine production, and to investigate its effect over an *in vivo* model of type 1 diabetes (T1D).

**Experimental procedure:** Splenocytes and sorted CD4<sup>+</sup>/CD8<sup>+</sup> from wild type C57BL/6 mice were cultured to determine activation, IFN- $\gamma$  release and T-cell proliferation after polyclonal stimulation. NOD (non-obese diabetic) mice were used to study the effects of orally administered extract on glycemia, insulinitis stages and perforin-1 (PRF-1)/granzyme-B (GRZ-B) expression.

**Results:** In primary cultures, the plant extract impairs T cell activation, decreases IFN- $\gamma$  release, and reduces proliferation after different polyclonal stimuli. *In vivo*, PsAE improves NOD mice glycemic levels and T1D progression by diminution of pancreas insulinitis and reduction of PRF-1 and GRZ-B mRNA expression. To our knowledge, this is the first report characterizing the therapeutic properties of PsAE on T cell activation.

**Conclusion:** The current work provides evidence about *in vitro* and *in vivo* immunosuppressive effects of PsAE and promotes this plant extract as a complementary and alternative treatment in autoimmune T-cell mediated diseases as T1D.

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**Abbreviations:** Disease, Metabolic disorder; Experimental design, Herbal medicine; Treatment with natural extracts, Dietary therapy.

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### 1. Introduction

Experimental approaches for improving T cell mediated diseases have been considerably developed in the last years.<sup>1</sup> Available treatments for autoimmune diseases are based on immunosuppressant drugs, such as methylprednisolone, azathioprine, hydroxychloroquine and mycophenolate mofetil; however, these therapies may not always have the desired efficiency.<sup>2</sup> The use of these drugs significantly improves the clinical output of illnesses but they are also associated with considerable side effects, such as major risks to infections, gonadal failure and bone marrow

**List of abbreviations**

CAM	complementary and alternative medicine
CFSE	(5-(and-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester)
ConA	concanavalin A
FACS	Fluorescence-activated cell sorting
FBS	fetal bovine serum
GRZ-B:	granzyme B
HPRT1	hypoxanthine-guanine phosphoribosyltransferase
IFN- $\gamma$ :	gamma-interferon
MFI	mean fluorescence intensity
NOD	non-obese diabetic mice
PBS	phosphate-buffered saline
PRF-1	perforin 1
PsAE	<i>P. strombulifera</i> aqueous extract
T1D	Type 1 diabetes

depression.<sup>3</sup> In recent years, the biological therapies based on T cell blocking with monoclonal antibodies are getting more common in clinical practice; however, they are expensive and their efficacy does not reach the expected results.<sup>4,5</sup> In relation to the natural products, only a few plant extracts are reported to be effective in the treatment of autoimmune diseases; while the aqueous extract of *Viola tricolor* has a long story documented in the Pharmacopoeia of Europe,<sup>6</sup> the water extracts derived from *Berberis* spp. and *Trypterigium wilfordii* which have been used in the Chinese Medicine.<sup>7,8</sup> Therefore, safer, alternative, more specific and economical therapies are on demand for T cell mediated diseases, such as rheumatoid arthritis, transplantation, and type 1 diabetes (T1D).<sup>9,10</sup> In consequence, identifying new products with tolerogenic properties on T cell responses would be crucial for developing alternative therapies and enhancing the efficacy of current clinical treatments.

Many drugs used in immunotherapy have been provided by nature. The contribution of natural products to the treatment of immune mediated diseases is evidenced by the identification of several NF $\kappa$ B inhibitors.<sup>11,12</sup> Although East Asian countries have a vast history of medicinal achievements, that keeps growing until today; in Argentina, the study of products obtained from regional plants is emerging and has been proclaimed a national scientific priority.<sup>13</sup> In particular, there are several species of native plants in Mendoza (Argentina) with folkloric reports of anti-inflammatory uses but none of them has been thoroughly studied with respect to its suppressive properties over lymphocytes proliferation or function.

*Prosopis strombulifera* (Lam.) Benth, commonly known as “retortuño”, is a rhizomatous shrub native from the northern and central zones of Argentina. The ethnopharmacological reports indicate that the plant has been used by the Huarpe pre-Columbian tribe as astringent, anti-inflammatory, odontalgic and anti-diarrheic agent; interestingly, some of the others genus species, but not *P. strombulifera*, were reported by its folkloric uses in the treatment of hyperglycemia and diabetes.<sup>14</sup> Recent scientific studies demonstrated that *P. strombulifera* aqueous extract (PsAE), orally administered in drinking water, does not induce acute or subchronic toxicity in laboratory animals,<sup>15</sup> determines antitumoral effects in colorectal and melanoma tumors in C57BL/6wt mice,<sup>16</sup> reduces atherogenesis in C57BL/6j ApoE-KO mice,<sup>17</sup> and delays cutaneous leishmaniasis caused by *Leishmania amazonensis* in BALB/c mice.<sup>18</sup> Moreover, the complete secondary metabolites profile of PsAE was characterized by UHPLC-Q/Orbitrap/MS/MS

including simple organic and phenolic acids, procyanidins, flavonoids and oxylipins.<sup>16</sup> Between the 26 phytochemicals identified, some of the more abundant are: catechin, kaempferol glucoside, myricitrin 3-O-glucoside, naringoside, rutin and xilonic acid. Some of these phytochemicals were previously reported due to their immunomodulatory and antidiabetic activities.<sup>19–24</sup>

In accordance to the mentioned precedents, the aim of this study was to evaluate the tolerogenic effect of PsAE during *in vitro* T cell activation and to investigate its effect over an *in vivo* model of T1D; promoting this herbal extract as a potential immunosuppressive product useful for T cell mediated diseases.

## 2. Material and methods

### 2.1. *P. strombulifera* collection and aqueous extracts preparation

Plant material was collected in Mendoza, Argentina (33° 44' 10" S, 68° 55' 21" 30.5" W) and a voucher specimen was deposited in the Mendoza Ruiz Leal herbarium (MERL 61824). The leaves of *P. strombulifera* were used for preparing the aqueous extract as indicated in Persia et al., 2020.<sup>16</sup>

### 2.2. Animals and in vivo procedures

The mice were bred and housed in the animal facility of Instituto de Medicina y Biología Experimental de Cuyo, Mendoza, Argentina in a light-dark cycles from lights on 6:00 a.m. to 10:00 p.m. and room temperature between 22 and 24 °C. Mice chow (Cargill, Córdoba, Argentina) and liquids were available *ad libitum*. Weight gain, drinking water and chow consumption were registered thrice a week. Two different sets of animals were used in the current work.

#### 2.2.1. Adult female C57BL/6 mice, 5–6 weeks old ( $n = 10$ ) were used to obtain spleens for primary culture and cytometry

2.2.2. The NOD mouse is a TD1 autoimmune model characterized by hyperglycemia, T cell infiltration of pancreatic islets and progressive insulin decrease. Due to NOD background and its polygenic genotype, this strain displays defects and flaws in T cells tolerance and antigen presentation.<sup>25</sup> In the current work, 6-weeks-old female mice were used to evaluate the effect of PsAE in T1D development during 10 weeks. These animals were separated in 2 groups ( $n = 10$  per group). One group received 150 mg/animal/day of PsAE treatment diluted in drinking water. This treatment concentration was established according to Hapon *et al.* (2014) as the maximum nontoxic reported dose.<sup>15</sup> The other group of animals was considered as control (0 mg/animal/day of PsAE) and were only provided drinking water during the experiment. In both groups, the serum glucose levels were monitored weekly during the 10 weeks of the experiment by a blood sample obtained from the tail vein using an Accu-Check performa blood glucose meter (Roche Diagnostics, Mannheim, Germany).

The animal maintenance and handling were performed in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health. All procedures were approved by the Institutional Animal Care and Use Committee of School of Medical Science, Universidad Nacional de Cuyo: protocol approval N° 89/2016 to C57BL/6 mice described in 2.2.1; and protocol approval N° 103/2017 to NOD mice described in 2.2.2.

#### 2.3. Splenocytes obtaining and determination of PsAE concentration used in treatments

Spleens from untreated C57BL/6 mice were harvested and minced in phosphate-buffered saline (PBS), supplemented with 5%

FBS (Internegocios, Argentina), until a homogeneous cell suspension was reached. Splenocytes were seeded at  $1 \times 10^6$  cells per well in a 24 well plate using RPMI1640 medium (GIBCO, USA), supplemented with 10% FBS, 100 IU of penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin (GIBCO, USA). The aqueous extract was added to the culture media at doses of 0–10  $\mu\text{g}/\text{mL}$  (0, 0.62, 1.25, 2.5, 3.5, 5, 7.5 and 10  $\mu\text{g}/\text{mL}$ ). After 96 h, in 5%  $\text{CO}_2$ , at 37 °C, the cell number and viability were determined by trypan blue exclusion assay counted in a Neubauer haemocytometer chamber. The highest dose that did not affect cell viability (2.5  $\mu\text{g}/\text{mL}$ ) was selected to study the immunomodulatory effects of PsAE *in vitro* (see below).

#### 2.4. Polyclonal stimulation assays

Isolated splenocytes were seeded in supplemented RPMI 1640 at  $1 \times 10^6$  cells per well in a 24 well plate. In treated groups, PsAE (2.5  $\mu\text{g}/\text{mL}$ ) was administered for 2 h. Thereafter, media was changed and splenocytes were stimulated by ConA (1  $\mu\text{g}/\text{ml}$ ; Sigma-Aldrich, US) or A23187 calcium ionophore (1  $\mu\text{g}/\text{mL}$ ; Sigma-Aldrich, US), for 24 h. In some experiments,  $\text{CD4}^+$  and  $\text{CD8}^+$  T cells from spleen cell suspension were purified by fluorescence-activated cell sorting (FACS), see below point 2.5. Next, the purified cells were incubated with agonist anti-mouse CD3 (clone 17A2 BD biosciences, US), and CD28 (anti-mouse CD28; clone 37.51. BD biosciences, US) agonists monoclonal antibodies for 24 h in a 96 well plate. The 96 well culture plate was previously bound with 100  $\mu\text{l}$  of anti-CD3 (1  $\mu\text{g}/\text{ml}$ ) and anti-CD28 (1  $\mu\text{g}/\text{ml}$ ). After that, cells were harvested, washed and stained with FITC-conjugated anti-CD4 (anti-mouse CD4-FITC/PE; clone GK1.5. BD Biosciences, US), anti-CD8 (anti-mouse CD8-PE/APC; clone 53–6.7. BD Biosciences, US), PE-conjugated anti-CD69 (anti-mouse CD69-PE/APC; clone H1.2F3. BD biosciences, US). In some cases, supernatants were stored at  $-80^\circ\text{C}$  to evaluate the presence of  $\text{IFN-}\gamma$ . The spleen cells or sorted T cells were cultured in triplicate wells by each experiment, and three independent experiments were performed.

#### 2.5. Isolation of $\text{CD4}^+$ and $\text{CD8}^+$ cells by FACS

To obtain  $\text{CD4}^+$  and  $\text{CD8}^+$  T cells, splenocytes were stained using FITC and PE conjugated anti-mouse, CD4 and CD8 as indicated in point 2.4. Then, cells were acquired and sorted in FACS Aria III cell sorter (BD Bioscience, San Diego, CA, USA) at the National University of Cuyo, School of Medical Sciences. The purity of the sorted populations was more than 93% for both subsets.

#### 2.6. Enzyme-linked immunosorbent assay (ELISA)

For  $\text{IFN-}\gamma$  detection in culture supernatants, sandwich ELISA was performed as described by the manufacturer (BD Biosciences, San Diego, CA, US) and optical density (OD) at 450 nm was measured on a microplate reader.

#### 2.7. Measurement of proliferative responses by CFSE dilution

Cell proliferation was assessed by CFSE (5-(and-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester) labeling of splenocytes, following the manufacturer instructions (Thermo Fischer, US). Briefly, freshly isolated spleen cells were stained with CFSE Cell Tracer Kit (1  $\mu\text{M}$ ) for 5 min at 37 °C. Then, labeled cells were washed 3 times in supplemented culture medium and quantified. Five hundred thousand cells per well were seeded into 96-well U-bottomed plates in a total volume of 200  $\mu\text{l}$  supplemented RPMI1640 medium. Then, cells were stimulated using ConA (1  $\mu\text{g}/\text{mL}$ ) for 4 days, in 5%  $\text{CO}_2$ , at 37 °C. The PsAE treatment was added to the culture medium in a dose response manner at 0, 0.62, 1.25 and

2.5  $\mu\text{g}/\text{mL}$ . At the end of the stimulation period, cells were washed in PBS and acquired in a FACS Aria III flow cytometer. Total proliferation is the percentage of CFSE low stained cells relative to CFSE stained cells from control untreated cultures.

#### 2.8. Degranulation assay

$\text{CD8}^+$  T cell degranulation was determined by CD107a staining (clone 1D4B from BD Pharmigen US) by flow cytometry. Briefly, freshly isolated spleen cells were cultured in the presence of 3  $\mu\text{g}/\text{mL}$  rat anti mouse CD107a antibody conjugated with APC, PMA 50 ng/mL and ionomycin 750 ng/mL. After 4 h, cells were harvested, stained with anti mouse CD8 conjugated with FITC and acquired in a FACS Aria III flow cytometer.

#### 2.9. Insulinitis quantification

At the end of the glycaemic control assay (week 10, see point 2.2.2.), NOD mice were sacrificed and their pancreas were dissected, fixed in formalin, embedded in paraffin and stained with hematoxylin and eosin. For each mouse, 6 sections at different depths of the pancreas were examined and 5–10 islets/sections were graded for insulinitis. The quantification was performed according to the area of an islet infiltrated by lymphocytes, as follows: 0% infiltration = grade 0, periinsulinitis and up to 10% infiltration = grade 1, 10–49% infiltration = grade 2, 50–74% infiltration = grade 3 and 75–100% infiltration = grade 4. A minimum of 50 islets were scored for each mouse.

#### 2.10. RNA isolation and RT-real time PCR analysis

Spleen tissues from control and PsAE mice were used to obtain total RNA. It was isolated using TRIzol<sup>®</sup> reagent (Invitrogen, 15596026) according to the manufacturer protocol. Total RNA was reverse transcribed, using random hexamer primers and Moloney murine leukemia virus retrotranscriptase (Invitrogen-Life Technologies, USA). Real-time qPCR was performed with cDNA samples and EVA Green (Biotium, Hayward, CA) using a Rotor-Gene 6000 Series Software version 1.7 (Corbett). The samples were amplified in triplicate. The oligonucleotide primers (OligoLTA, Thermo Fischer Scientific) used were: perforin (PRF-1) sense: 5'-GAGAA-GACCTATCAGGACCA-3' and antisense 5'-AGCCTGTGGTAAGCATG-3'; granzyme B (GRZ-B) sense: 5'-CCTCCTGCTACTGCTGAC-3', and antisense: 5'-GTCAGCACAAGTCTCTC-3; and, HPRT1 (hypoxanthine-guanine phosphoribosyltransferase) sense: 5'-GTTGGATACAGGCCAGACTTTGTTG-3', and antisense; 5'-GATTCAACTTGGCTCATCTTAGGC-3'. PCR reactions were initiated with 5 min incubation at 95 °C, followed by 40 cycles of: 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s mRNA expression levels of PRF-1 and GRZ-B were normalized to the HPRT1 reference gene. To compare the relative expression between treatments, the mRNA levels were expressed as a ratio using the delta-delta method. Relative expression was calculated as  $2^{-\Delta\Delta\text{Ct}}$ .

#### 2.11. Statistics

All data are expressed as mean  $\pm$  standard error (SEM) and analyzed using GraphPad Prism 6.0 software. (Graph Pad Software, Inc., San Diego, CA, USA). For statistical analyses, Student's T-test was used to compare groups and values of  $p \leq 0.05$  were considered statistically significant.

### 3. Results

#### 3.1. PsAE decreases CD69 expression after polyclonal activation

One of the most important features of systemic immunosuppressive treatment is to prevent cellular activation keeping T cells silent.<sup>26</sup> Upon activation, T cells enhance the expression of different molecules including lymph node homing receptors such as CD69 to ensure full signaling from TCR/CD3 complexes that may lead to clonal expansion.<sup>27</sup> To evaluate whether PsAE treatment could decrease immune cell activation, spleen cells from C57BL/6 mice were exposed to ConA or A23187, for 24 h. As expected, both stimuli were able to induce CD69<sup>+</sup> expression. However, when PsAE (2.5 µg/mL) was present in the culture media, the activation was reduced (Fig. 1A). In splenocytes activated with ConA, the percentage of CD69<sup>+</sup> cells decreased from 20.6 ± 1.3% to 3.5 ± 0.1% in the presence of PsAE; while in A23187 exposed cells, PsAE reduced the percentage of CD69<sup>+</sup> cells from 11.4 ± 0.2 to 1.8 ± 0.2%. In both cases, the reduction was statistically significant (p < 0.0001) (Fig. 1B). In accordance with these results, PsAE treatment decreases immune cell activation measured by the expression of CD69.

#### 3.2. PsAE reduces CD69 expression of isolated CD4<sup>+</sup> and CD8<sup>+</sup> T cells after CD3/CD28 stimulation

Next, to determine whether PsAE could suppress CD4<sup>+</sup> and CD8<sup>+</sup> T cell activation, we used spleen cell cultures stimulated with ConA to induce polyclonal activation. To evaluate if PsAE treatment ameliorates T cell activation independently of accessory cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were purified by FACS cell sorting. After sorting, the purified cells were treated with PsAE (2.5 µg/mL) for 2 h, then they were stimulated with antiCD3/CD28 monoclonal antibodies for 24 h and analyzed by flow cytometry. We found that CD4<sup>+</sup> and CD8<sup>+</sup> T cells were immunosuppressed by PsAE due to the significantly decreased level of activation, measured by the expression of CD69. While CD4<sup>+</sup> cell activation was reduced for PsAE from

59.7 ± 0.5 to 20.1 ± 0.7%, the CD8<sup>+</sup> population was reduced from 54.8 ± 2.0 to 13.9 ± 0.5 (Fig. 2) (p < 0.0001). Thus, our results confirm that PsAE directly prevents CD4<sup>+</sup> and CD8<sup>+</sup> T cell activation independently of accessory cells.

Additionally, we found that PsAE also prevents the induction of CD4 and CD8 expression in sorted CD4<sup>+</sup> and CD8<sup>+</sup> cells stimulated with CD3 and CD28 (Fig. 3A). In PsAE treated (2.5 µg/mL) and CD3/CD28 stimulated cells, the mean fluorescence intensity (MFI) of CD4 and CD8 were significantly reduced from 2322 ± 24.08 to 1656 ± 17.88 in CD4<sup>+</sup>; and from 2603 ± 42.63 to 1351 ± 31.20 in CD8<sup>+</sup> (p < 0.0001) (Fig. 3B).

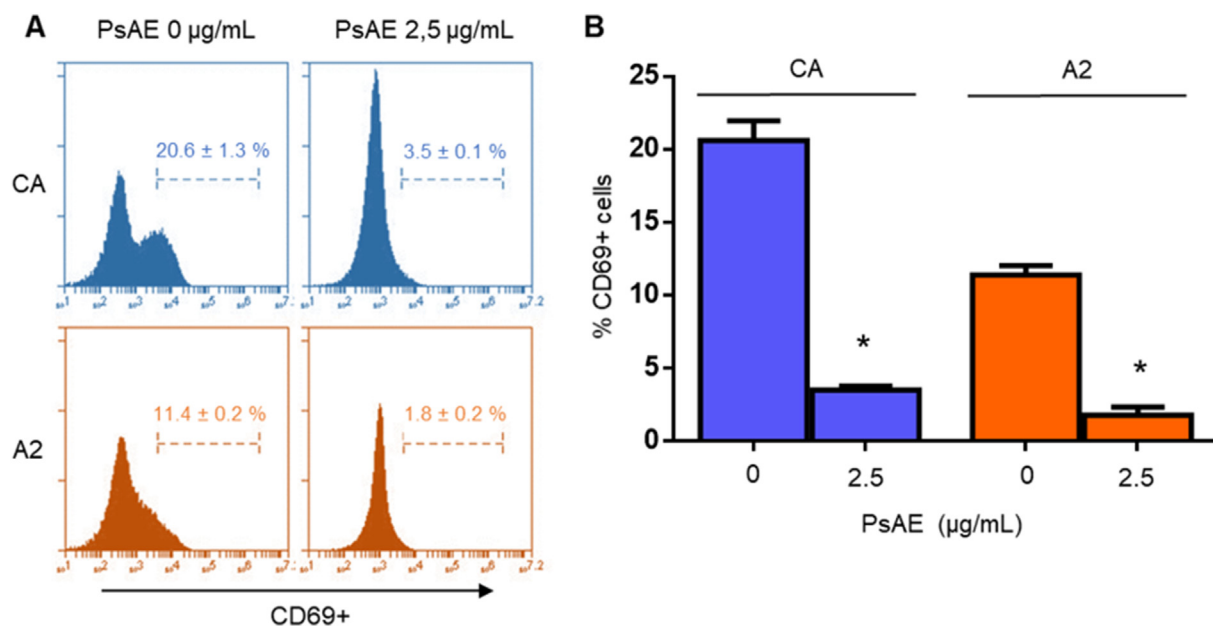
According to the results obtained, it is possible to indicate that CD4<sup>+</sup> and CD8<sup>+</sup> mice splenocytes treated with PsAE experienced marked cellular suppression after different stimulation procedures.

#### 3.3. PsAE diminishes IFN-γ cytokine release

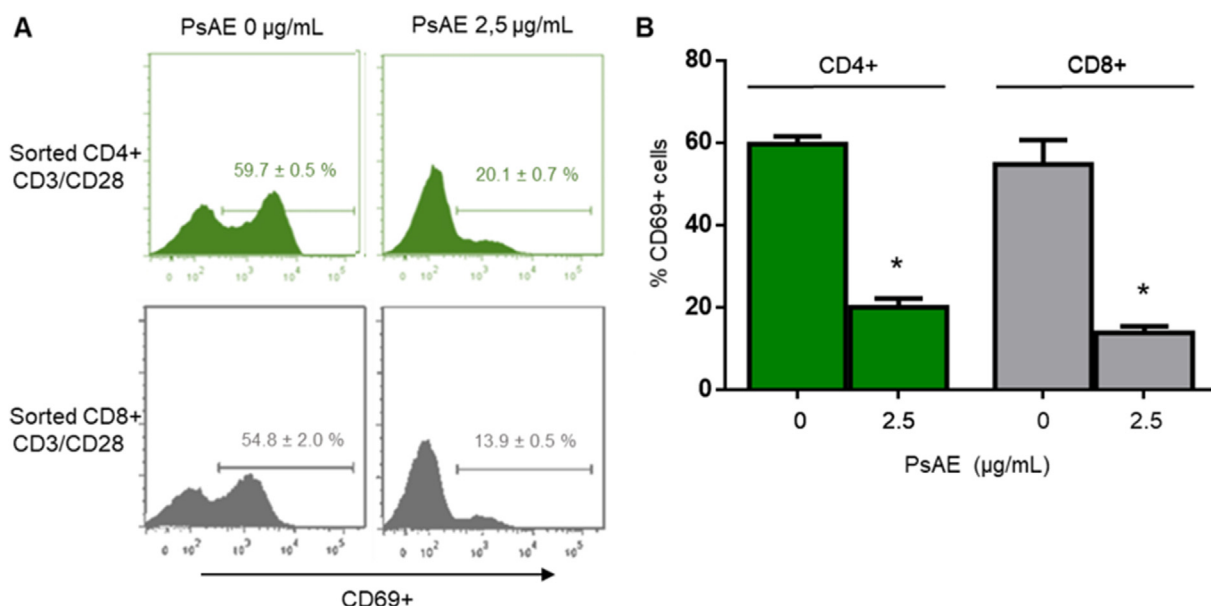
The finding that PsAE alters T cell activation suggests that immune function may also be affected. It is known that IFN-γ plays a crucial role in immune responses, inflammation and several autoimmune pathologies such as T1D.<sup>28,29</sup> To evaluate the contribution of PsAE in the suppression of effector immune cells, we measured IFN-γ levels in the culture supernatants of ConA stimulated spleen cells. The PsAE (2.5 µg/mL) treated cultures showed a significant reduction of IFN-γ release compared to untreated cultures. While untreated cells showed levels of 249.3 ± 53.3 pg/mL after ConA stimulation, the PsAE treated splenocytes evidenced a significant diminished level of cytokine release reaching 70.66 ± 4.5 pg/mL (p = 0.0078) (Fig. 4). These results confirm that the herbal extract interferes with the immune response at cellular and cytokine levels.

#### 3.4. PsAE exposure alleviates immune cell proliferation in a dose-response manner

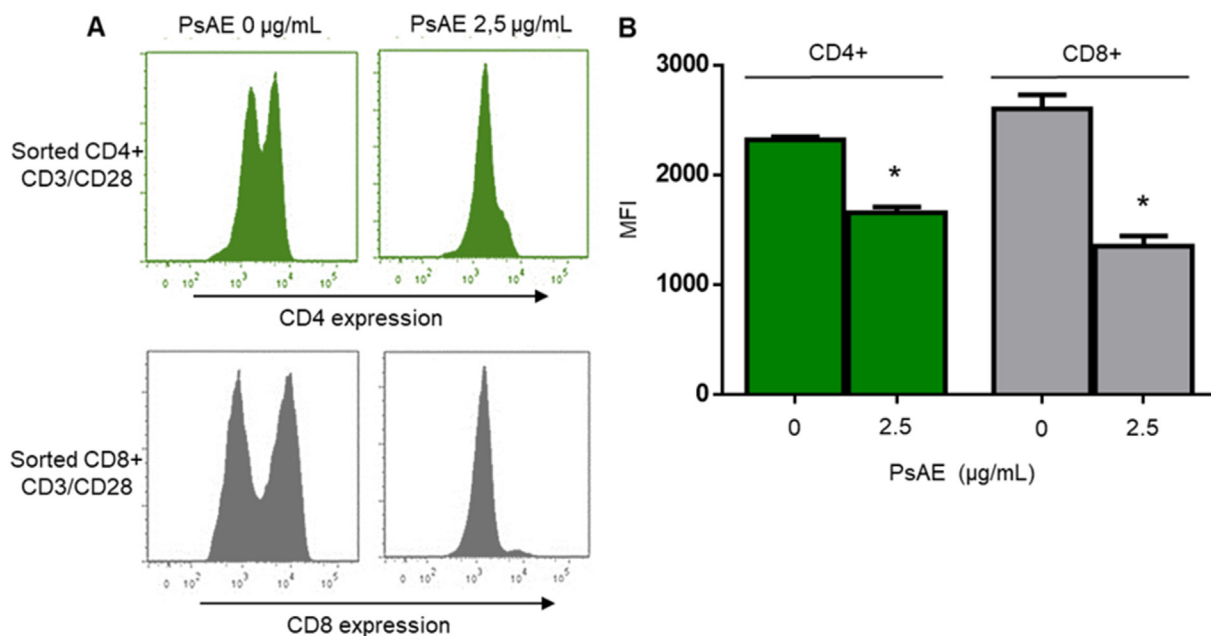
Different immunologic diseases course with lymphoproliferation and the proliferation management of T cells were reported as a



**Fig. 1.** PsAE decreases CD69 expression after polyclonal activation. In panel A, the histograms of CD69<sup>+</sup> cells show the changes induced by the PsAE in ConA (blue) and A23187 (orange) stimulated cultures. In panel B, the percentage of activated CD69<sup>+</sup> under different conditions are compared. The data shown are the mean ± SEM of three independent experiments. Statistical significance, Student T test (p < 0.0001) are indicated by the asterisk (\*). In the graph, CA: ConA and A2: A23187.



**Fig. 2.** PsAE reduces CD69 expression of isolated CD4<sup>+</sup> and CD8<sup>+</sup> T cells after CD3/CD28 stimulation. Panel A represents the histograms of sorted CD4<sup>+</sup> cells (green) and CD8<sup>+</sup> cells (gray) and the changes induced by PsAE treatment (2.5 µg/ml). In panel B, bars represent the percentage of activated (CD69<sup>+</sup>) CD4<sup>+</sup> (green) and CD8<sup>+</sup> cells (gray) with none and 2.5 µg/ml of PsAE. The data shown are the mean ± SEM of three independent experiments. Statistical significance, Student T test (p < 0.0001) are indicated by the asterisk (\*).



**Fig. 3.** PsAE prevents the induction of CD4 and CD8 expression of sorted CD4<sup>+</sup> and CD8<sup>+</sup> cells. The panel A shows representative histograms of sorted CD4<sup>+</sup> cells (green) and CD8<sup>+</sup> cells (gray) control (untreated, PsAE 0 µg/mL) and treated with PsAE (2.5 µg/mL), both stimulated with CD3 and CD28. In panel B, bars represent the mean fluorescence intensity (MFI) of CD4<sup>+</sup> (green) and CD8<sup>+</sup> T cells (gray). The data shown are the mean ± SEM of three independent experiments. Statistical significance, Student T test (p < 0.0001) are indicated by the asterisk (\*).

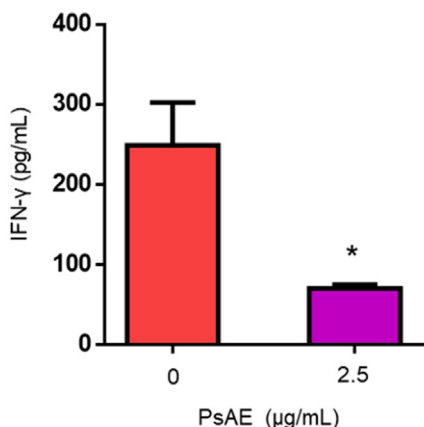
valuable therapeutic approach in immune mediated and autoimmune diseases, including T1D.<sup>30</sup> The finding that PsAE affects immune cell activation and IFN-γ release suggests that proliferation may also be disturbed. To determine whether PsAE prevents immune cell proliferation, murine spleen cells were stained with CFSE and treated in a dose response manner with PsAE, in the presence of ConA, for 96 h. To precise changes in cell number and viability, the quantification was realized by Trypan blue staining in a haemocytometer chamber. We found that PsAE decreased immune cell

count, proliferation and viability in a dose-response manner (Fig. 5A–D). Thus, PsAE prevention of splenocyte polyclonal proliferation suggests that the extract would be a potential natural agent to treat lymphoproliferation in T cell immune mediated diseases.

### 3.5. Oral administration of PsAE ameliorates T1D in NOD mice

To determine if the immunomodulatory effects observed *in vitro*





**Fig. 4.** PsAE diminishes IFN- $\gamma$  cytokine release. Bars represent the production of IFN- $\gamma$  after ConA activation in control (untreated, red) and PsAE treated (2.5  $\mu$ g/ml, purple) splenocyte cultures. The data shown are the mean  $\pm$  SEM of three independent experiments. Statistical significance, Student T test ( $p < 0.01$ ) are indicated by the asterisk (\*).

by PsAE could ameliorate T1D *in vivo*, we studied the effect of PsAE administered in drinking water to NOD mice, as a model of T-cell dependent autoimmune disease. This animal model is widely used to study the autoimmune T1D triggered by the pancreas infiltration and T cell-mediated destruction of the insulin-producing beta cells.<sup>31</sup>

When the experiment started, the controls (0 mg/animal/day) and PsAE (150 mg/animal/day) groups of NOD mice presented similar glucose levels: 108  $\pm$  4.3 mg/dL in controls and 112.2 mg/dL  $\pm$  3.3 in the PsAE treated. Thereafter, glycaemia rose in both groups of animals, but this increase was markedly higher in the control group until the difference was statistically significant after week 9. At this point, glycaemia values in controls were 295.8  $\pm$  37.83 mg/dL while in the treated group was 146.8  $\pm$  11.12 mg/dL ( $p = 0.0014$ ) (Fig. 6). In relation to TD1 incidence, considering that mice are diabetic when blood glucose levels are above 250 mg/dL for 2 consecutive blood glucose tests<sup>32</sup>; in the control group, 3 animals became diabetics at week 7 and, at the end of the experiment, 90% of animals developed diabetes. On the other hand, in the PsAE treated group, only 1 animal developed diabetes at week 9. In respect to the body weight, a marked but not statistical difference was found; while PsAE treated animals evidenced an increase of 4.33  $\pm$  1.6 g, the control non-treated animals showed a slight diminution of 1.07  $\pm$  1.2 g. Finally, there was no difference in liquid or chow consumption, with averages of 5.02  $\pm$  0.9 mL and 3.8  $\pm$  1.2 g, respectively.

### 3.6. PsAE improves T1D by diminution of beta-islets lymphocyte infiltration

The T1D hallmark pathological lesion is a heterogeneous inflammatory cell infiltrate recognized as insulinitis.<sup>33</sup> To study if PsAE interferes with a lymphocyte infiltration and consequent beta islets destruction, we next determine the pancreas insulinitis grades by optical microscopy. After 10 weeks of PsAE treatment, the insulinitis analysis revealed that pancreatic islets had lower levels of infiltration than control groups. The measure of lymphocyte infiltration in the treated group evidenced different stages of insulinitis; with 32.6  $\pm$  4.6%, grade 1 resulted the predominant stage in these animals. On the other hand, in the control group, the most frequent finding was the insulinitis grade 4 (84.9  $\pm$  9%) which was sporadically accompanied by total destruction of the acinus (Fig. 7). The presented results evidence that PsAE oral administration has a

protector effect on pancreatic beta-cells, impairing lymphocyte infiltration and their consequent destruction.

### 3.7. PsAE mitigates mRNA expression levels of PRF-1 and GRZ-B

CD4<sup>+</sup> and CD8<sup>+</sup> T cells participate in insulinitis and beta-cells destruction by the release of cytotoxic molecules. Specifically, PRF-1 and GRZ-B have been shown to play an integral part in the development of T1D.<sup>34,35</sup> To determine if the minor grade of insulinitis induced by PsAE is accompanied by a diminished genetic expression of PRF-1 and GRZ-B on immunocompetent cells, we next performed RT-qPCR on NOD mice spleens. After 10 weeks, the treatment with PsAE determined a minor grade of mRNA expression of both PRF-1 and GRZ-B in NOD mice spleen (Fig. 8). Whereas relative expression of PRF-1 mRNA was 5.40  $\pm$  1.5 in controls, in the treated group was significantly reduced to 1.11  $\pm$  0.4 ( $p = 0.019$ ). In the case of GRZ-B, controls also showed a significantly higher mRNA relative expression of 2.54  $\pm$  0.8 versus 0.67  $\pm$  0.3 in the treated group ( $p = 0.047$ ). To corroborate the suppressive properties of PsAE on the cytotoxic potential of CD8<sup>+</sup> T cells, we performed an *in vitro* degranulation assay based on CD107a staining upon polyclonal activation (Supplementary Fig. 1). We found that PsAE significantly decreased the percentage of CD107a<sup>+</sup> CD8<sup>+</sup> T cells compared to control cells ( $p = 0.018$ ). Together, these results indicate that PsAE may reduce T cells dependent cytolysis *in vivo*, collaborating to ameliorate insulinitis and T1D progression.

## 4. Discussion

T1D is a T cell-driven illness where CD4<sup>+</sup> and CD8<sup>+</sup> are involved in the disease development and progression. With a reported worldwide prevalence of 9.5 per 10,000 people and an incidence of 15/100,000 people-year, T1D is the most prevalent chronic metabolic disorder from children to younger adults.<sup>36,37</sup> The reported frequency of complementary and alternative medicine (CAM) use associated with T1D is between 18.6% and 56.4%, being herbal extracts the most widely used method.<sup>38–40</sup>

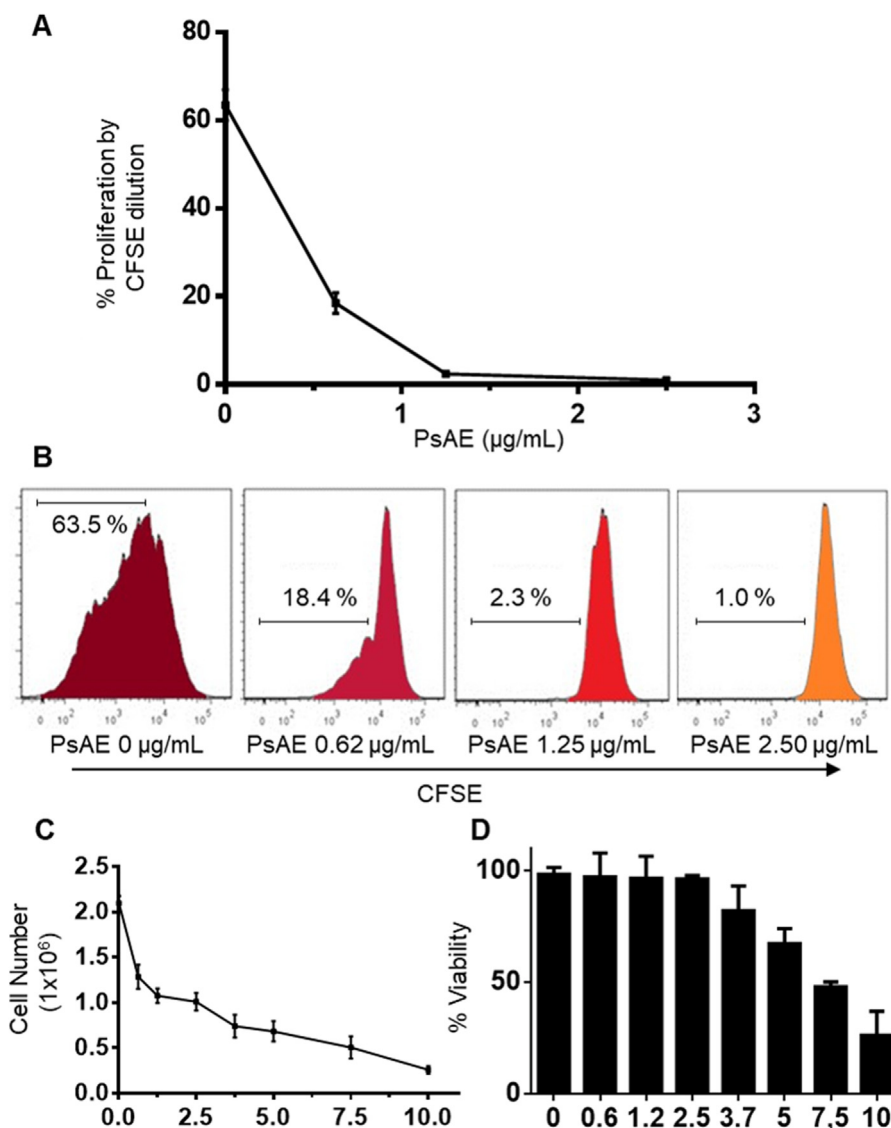
The current work demonstrates that PsAE decreases the expression of CD69<sup>+</sup> on splenocyte primary cultures after polyclonal stimulation. Due to PsAE decreases T cell activation after different activating stimuli, we propose that PsAE inhibits key signaling pathways involved in immune cell activation such as NFAT, AP-1 and/or NF $\kappa$ B activation. Our data also suggest that the anti-proliferative effects of PsAE over T cells could be the result of a blockade in early events during cell activation. In addition, the PsAE capacity to affect IFN- $\gamma$  release and to decline lymphocyte proliferation provide valuable biological properties to prevent and treat T1D; since in both, NOD mice and human disease, IFN- $\gamma$  plays a relevant role in T cell mediated  $\beta$  cell destruction.<sup>41,42</sup>

The precise mechanism related to PsAE immunosuppressive function over T cells could be difficult to determine. Among the phytochemicals of the aqueous extract phytochemicals they are excellent candidates to drive the tolerogenic and anti-inflammatory phenotype seen in experimental T1D. In accordance to our criterion, it is probably that different cellular mechanisms may be involved at the same time.

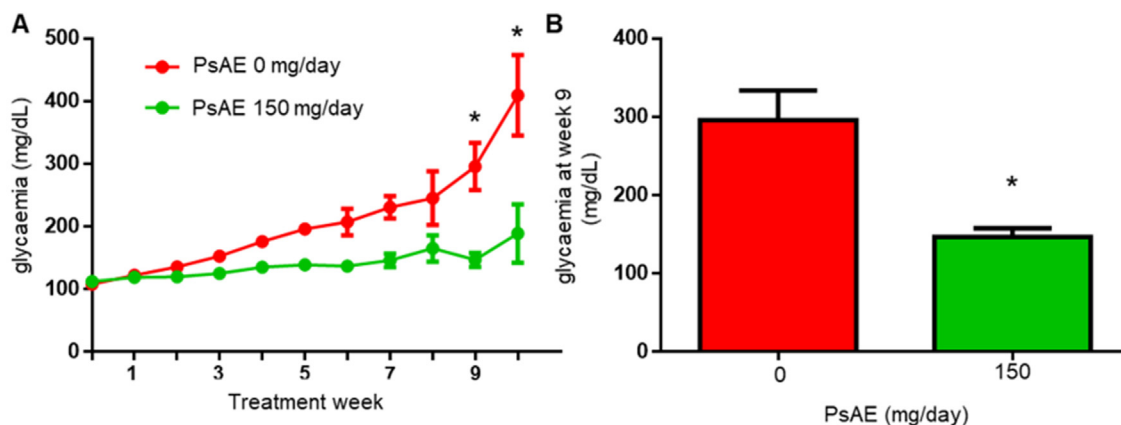
The protocatechuic acid is a phenolic acid identified in the PsAE which reduces the production of IFN- $\gamma$ , IL-6 and IL-8 by the suppression of NF- $\kappa$ B activation pathway.<sup>22</sup>

Procyanidins is other group of compounds present in PsAE that inhibit T cell activation and proliferation and diminishes IFN- $\gamma$  release<sup>19</sup>

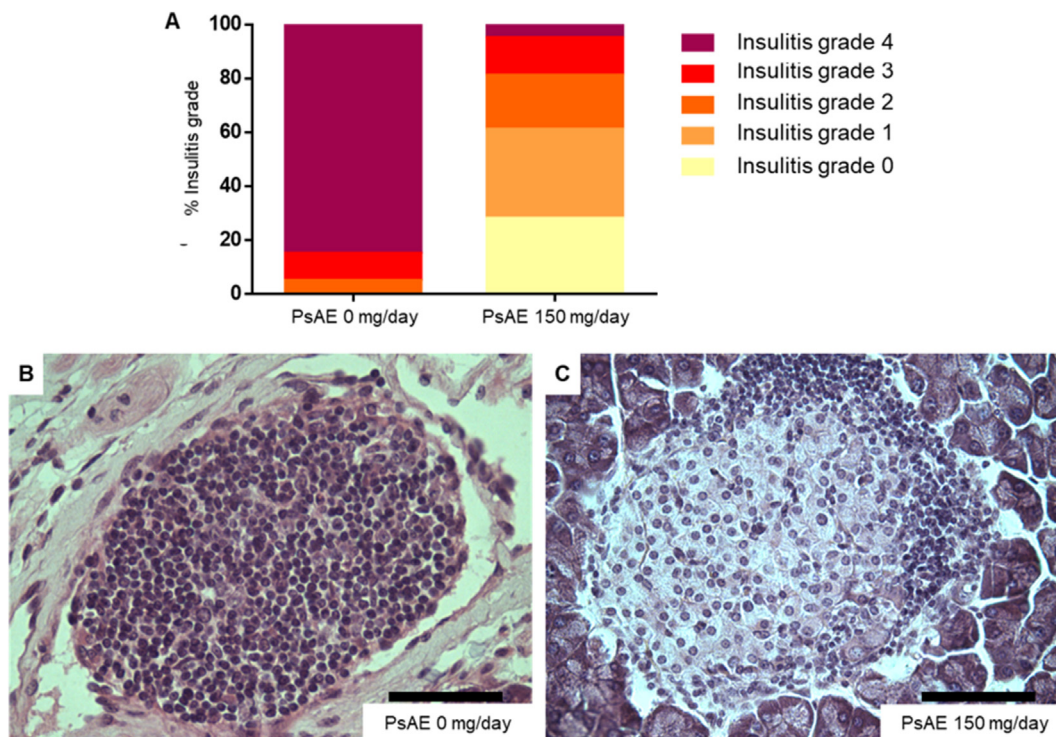
Among the flavonoid compounds, kaempferol is other PsAE component with the capacity to inhibit the development and the expansion of CD8<sup>+</sup> T cells, suppressing IFN- $\gamma$  and other cytokines



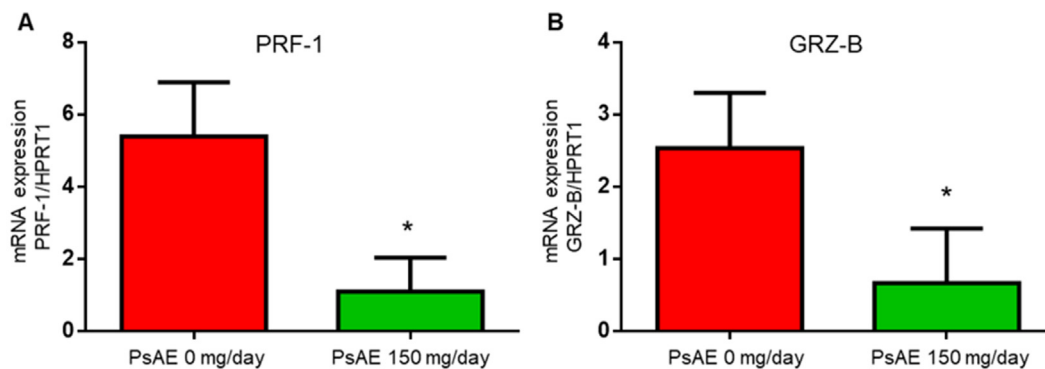
**Fig. 5.** PsAE exposure alleviates immune cell proliferation and viability in a dose-response manner. Panel A represents the percentage of ConA activated splenocytes measured by CFSE dilution after PsAE treatments (0, 0.62, 1.25 and 2.5 µg/mL). Panel B shows the representative histograms of PsAE dose response analyzed by flow cytometry. Panels C and D present the dose treatment related changes in cell number and viability, respectively. The data shown are the mean ± SEM of three independent experiments.



**Fig. 6.** Oral administration of PsAE ameliorates T1D in NOD mice. Panel A shows glycaemia levels (mg/dL) during 10 weeks. Panel B displays the difference in glucose levels among controls (PsAE 0 mg/day, red) and PsAE treated mice (green), at week 9. Each group consist of n = 10 animals. The data shown represent mean ± SEM values. The asterisks (\*) in panel A indicates statistical between groups; in panel B the statistical significance is also indicated by the asterisk (\*) (Student T test, p = 0.0014).



**Fig. 7.** PsAE improves T1D by diminution of beta-islets lymphocyte infiltration. The histological analysis of beta pancreatic islets of controls (0 mg/animal/day) and PsAE treated (150 mg/animal/day) NOD mice evidenced a notorious reduction in the insulinitis grades quantified by islet lymphocyte infiltration (panel A). Lower panels show 2 representative insulinitis grades. In B, the most frequent insulinitis (grade 4) found in a control group; and in C, the most frequent peri-insulinitis (grade 1) observed in a PsAE treated animals. Scale bar indicates 40  $\mu$ m.



**Fig. 8.** PsAE mitigates mRNA expression levels of PRF-1 and GRZ-B. Spleen mRNA relative expression of cytotoxic molecules PRF-1 and GRZ-B, measured by RT-qPCR in the control (0 mg/animal/day, red bars) and PsAE treated (150 mg/animal/day, green bars) NOD mice. Panel A shows the changes observed in PRF-1 and panel B the changes in GRZ-B, both calculated in relation to HPRT1 housekeeping expression. The data shown are the mean  $\pm$  SEM of n = 10 animals/group, processed in triplicated. Statistical significance, Student T test ( $p < 0.05$ ) are indicated by the asterisk (\*).

production indicating its potential effect in cell-mediated immune diseases.<sup>43</sup> Moreover, kaempferol prevents JNK phosphorylation, NF-KB-nuclear translocation upon T cell activation in which a direct interaction with MRP-1 transporter would be involved.<sup>44</sup> Rutin is another present flavonoid in PsAE that inhibits proliferation of splenocytes and thymocytes under ConA stimulation, and decreases the IFN- $\gamma$  production by the modulation of NF-KB production.<sup>45</sup>

Additionally, some flavanols present in PsAE such as catechin, galliccatechin and epigallocatechin were reported to affect CD8<sup>+</sup> T cells physiology and prevent chronic inflammation delaying the onset of T1D in non-obese diabetic (NOD) mice.<sup>24,46</sup> Specifically, it was found that epigallocatechin reduces T cell activation processes

by preventing ERK and AP-1 activation.<sup>47</sup>

In the NOD mice model, our findings confirm the PsAE bioavailability and its beneficial effects over glycaemia levels control and insulinitis development. The fact that PsAE mice displayed reduced insulinitis, observed by histological studies, suggests that immune cells, and most probably autoreactive T cells, do not reach islets. This could be due to different mechanisms including a reduced T cell activation, adhesion molecules expression, differentiation to effectors functions, or recruitment to target organs. Our findings are consistent with reported data on NOD mice where a diminished CD8<sup>+</sup> and CD4<sup>+</sup> T cell infiltration of the pancreas upon aqueous leaf extract of *Passiflora alata* treatment follows a lower destruction of insulin producing cells inside pancreatic islets.<sup>48</sup>



Summarizing, in the present work, the reported PsAE effects on splenocyte primary cultures and NOD mice demonstrate that this plant extract has tolerogenic properties effective that control T1D establishment and progression. Moreover, these results pose PsAE research and treatment as a potential effective therapy in T cell-mediated diseases as well as in transplantation through the inhibition of T cell activation. In this context, complementary treatment with PsAE, might be effective to address multiple immune and metabolic deficits of T1D, by reducing potential side effects associated with the conventional therapy, and to lengthen the immune modulation process over time.

## 5. Conclusion

In the current work, we provide evidence about the *in vitro* and *in vivo* immunosuppressive effects of PsAE. Altogether, the current findings promote PsAE as a plant extract valuable to T1D complementary and alternative treatment, as well as other diseases based in autoimmune T-cell pathogenesis. Future perspectives need to address the entire effect of PsAE on *in vivo* immune cell activation, to consider evidence related to the pharmaceutical presentations, to analyze the herbal medicine cost-benefit production and to study the combination or interactions with other conventional drugs available on the market.

## Founding sources

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## Declaration of competing interest

The authors declare that there is no conflict of interests to disclose.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jtcme.2022.10.001>.

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