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Prevention of experimental diabetes by *Uncaria tomentosa* extract: Th2 polarization, regulatory T cell preservation or both?

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ABSTRACT

Ethnopharmacological relevance: Uncaria tomentosa (Willd.) DC (Rubiaceae) is a species native to the Amazon rainforest and surrounding tropical areas that is endowed with immunomodulatory properties and widely used around the world. In this study we investigated the immunomodulatory potential of *Uncaria tomentosa* (UT) aqueous–ethanol extract on the progression of immune-mediated diabetes.
Materials and methods: C57BL/6 male mice were injected with MLDS (40 mg/kg) and orally treated with UT at 10–400 mg/kg during 21 days. Control groups received MLDS alone or the respective dilution vehicle. Pancreatic mononuclear infiltrate and β-cell insulin content were analyzed by HE and immunohistochemical staining, respectively, and measured by digital morphometry. Lymphocyte immunophenotyping and cytokine production were determined by flow cytometry analysis.
Results: Treating the animals with 50–400 mg/kg of UT caused a significant reduction in the glycemic levels, as well as in the incidence of diabetes. The morphometric analysis of insulitis revealed a clear protective effect. Animals treated with UT at 400 mg/kg presented a higher number of intact islets and a significant inhibition of destructive insulitis. Furthermore, a significant protection against the loss of insulin-secreting presented β-cells was achieved, as observed by a careful immunohistochemical evaluation. The phenotypic analysis indicated that the groups treated with higher doses (100–400 mg/kg) presented $CD4^+$ and $CD8^+$ T-cell values similar to those observed in healthy animals. These same higher doses also increased the number of $CD4^+CD25^+Foxp3^+$ regulatory T-cells. Moreover, the extract modulated the production of Th1 and Th2, with increased levels of IL-4 and IL-5.
Conclusions: The extract was effective to prevent the progression of immune-mediated diabetes by distinct pathways.

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1. Introduction

Immuno-mediated diabetes mellitus is a chronic disease with multifactorial etiology and whose incidence is exponentially increasing in developed nations since the 1950s (Lévy-Marchal et al., 1995; Zipris, 2009). The early stage of the disease is characterized by an inflammatory mononuclear infiltration into pancreatic islets, with the progressive loss of insulin-secreting β-cells (Atkinson and Eisenbarth, 2001). Although both humoral and cellular mechanisms are activated during the progression of the disease $CD4^+$ and $CD8^+$ T cells seem to be important final effectors of β-cell death (Pearl-Yafe et al., 2007). It has been demonstrated that the severity of insulitis is closely related to an increased expression of pro-inflammatory cytokines such as IFN-γ and TNF-α and a concomitant reduction in IL-4, IL-5 and IL-10 levels (Rabinovitch and Suarez-Pinzon, 1998). Furthermore, a fundamental role has been attributed to $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells on the regulation of immune tolerance and the self-reactive response during the progress of this disease (Chen et al., 2005; Wing and Sakaguchi, 2010).

In susceptible strains of mice, immune-mediated diabetes can be induced by multiple low-doses of streptozotocin (MLDS) (Reddy et al., 2008; Lin et al., 2010). This model is quite different from the type 2 diabetes model induced by a single high dose of streptozotocin. Several studies have shown that the injection of MLDS trigger an immune-mediated disorder characterized by a

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mononuclear inflammatory infiltrate within the pancreatic islets and the progressive loss of functional β-cells through the activity of self-reactive Th1 cells, macrophages and pro-inflammatory cytokines such as IFN-γ (Cetkovic-Cvrlje and Uckun, 2005). Peripheral changes such as downregulation of Th2-derived cytokines and inhibition of Treg cells were also demonstrated (Zdravkovic et al., 2009). One of the advantages of this model is the fact that hyperglycemia and insulitis are observed in a relatively short period of time (2–3 weeks after the last STZ injection) in a high percentage of animals (Paik et al., 1980; Santos et al., 2009).

In this sense, ideal immune-based interventions should be initiated before the onset of the disease or, at least, during the period when a reasonable number of β-cells is still present in the pancreas. Therefore, herbal immunomodulators could provide an interesting tool to prevent or to reduce the destruction of islet cells through the dynamic modulation of immunological functions.

Uncaria tomentosa (Willd.) DC (Rubiaceae), known as cat's claw, is a species native to the Amazon rainforest and surrounding tropical areas that is endowed with immunomodulatory properties. These properties include modulation of the cytokines TNF-α, IL-1β and IL-6 (Lemaire et al., 1999; Allen-Hall et al., 2007, 2010), enhancement of phagocytosis (Wagner et al., 1985), regulation of iNOS and NF-κB expression (Sandoval-Chacón et al., 1998) and the inhibition of lymphocyte proliferation (Akesson et al., 2003; Winkler et al., 2004). More recently, Reis et al. (2008) reported that the alkaloid fraction from *Uncaria tomentosa* bark caused a strong inhibition of TNF-α and IFN-γ production by human monocytes that were infected with type-2 Dengue virus. So far, the chemical analysis of the species revealed the presence of indole and oxindole alkaloids, proanthocyanidins, sterols, triterpenoids and carboxylalkyl esters (Sandoval et al., 2000; Akesson et al., 2005; Gonçalves et al., 2005; Heitzman et al., 2005). Individually or synergistically, these compounds contribute to the therapeutic properties of the species.

The present work was based on the hypothesis that the immunomodulatory properties of *Uncaria tomentosa* could prevent β-cell mass loss and therefore delay diabetes progression. We evaluated the effect of a chemically defined hydroethanolic extract of *Uncaria tomentosa* bark on diabetes development, cytokine production and number of peripheral $Foxp3^+$ Treg cells by employing the classic MLDS immune-mediated diabetes model.

2. Materials and methods

2.1. Plant material and extract chemical profile

An aqueous–ethanol 1:1 extract that was previously obtained (Valente et al., 2006) from the stem bark of a wild specimen of *Uncaria tomentosa*, collected in Cruzeiro do Sul, Acre, Brazil, and donated by Biosapiens Co., Brazil (voucher data in Miranda et al., 2003), was used for the assays. The pentacyclic oxindole alkaloid (POA) profile and content of this extract was determined using high-performance liquid chromatography (HPLC–UV-DAD–MS) as previously described (Pereira et al., 2008; Reis et al., 2008). Briefly, an alkaloid enriched fraction was obtained through classical acid–base partition from this aqueous–ethanol extract. The pentacyclic oxindole alkaloids (POA) present were identified by using a Lichrocart Lichrospher 5 μm, 125 mm × 4.6 mm i.d. column and UV-detector at 245 nm under the same conditions described by Laus and Keplinger (1994). The identification of the POA signals was made by comparison of their relative retention times to pteropodine with those described. In addition, the fraction was submitted to HPLC-DAD–MS analysis in the same chromatographic column using a mixture of 45% of CH_3CN/55% of aqueous 30 mM NH_4OAc solution, pH adjusted at 6.8–7.0 as mobile phase, isocratic mode, flow rate of 1.0 mL/min, at 60 °C and detection at 245 nm in order to monitor the ion $[M+1]^+$ at *m*/*z* 369 (Lopez-Avila et al., 1997) and the corresponding UV spectra (Mazzei, 2004). The determination of the total alkaloid content was achieved by HPLC–UV under the latter chromatographic conditions through external standardization relative to isopteropodine.

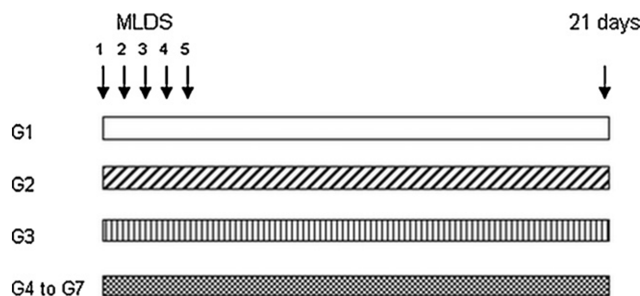

Fig. 1. Experimental protocol. Diabetes was induced by five sub-diabetogenic doses of streptozotocin or its dilution vehicle as follows. G1: alcohol 10% and citrate buffer; G2: *Uncaria tomentosa* extract 400 mg/kg and citrate buffer; G3: MLDS 40 mg/kg and alcohol 10%; G4–G7: MLDS 40 mg/kg and *Uncaria tomentosa* extract at 10, 50, 100 or 400 mg/kg, respectively. *n* = 8 animals/group. *Uncaria tomentosa* extract or its dilution vehicle (alcohol 10%) was administered by oral route. MLDS or its dilution vehicle (citrate buffer) was injected by intraperitoneal route.

2.2. Animals

Six weeks old pathogen-free C57BL/6 male mice were purchased from the Multidisciplinary Center of Biological Investigations (CEMIB, Campinas, São Paulo, Brazil) and housed in controlled environmental conditions (temperature 22 ± 2 °C, relative humidity 55 ± 20%, 12/12 h light–dark cycle and 4 daily exhaustion periods). All animals received Nuvilab CR-1 commercial food (Nuvital, Brazil) and water *ad libitum* and were submitted to a 2-week acclimation period before the experiment. Experimental protocols were approved by the Committee for Ethics in Animal Experimentation of the UNESP Medical School, São Paulo, Brazil (protocol number 635).

2.3. Diabetes induction and experimental design

The animals were randomized in groups of eight, according to the protocol presented in Fig. 1. Experimental diabetes was induced by five intraperitoneal (i.p) injections of streptozotocin (Sigma–Aldrich, St. Louis, MO, USA) at 40 mg/kg, during 5 consecutive days (Fallarino et al., 2010). *Uncaria tomentosa* extract was given by gavage during 21 consecutive days, beginning at the first streptozotocin injection. A control group injected with the streptozotocin vehicle (citrate buffer, pH 4.5) was treated with the *Uncaria tomentosa* diluent (alcohol 10%). Blood samples were collected from the retro-orbital plexus and glucose concentration was assessed on days 0, 12 and 21, using the Prestige LX Smart System Test-strips (Home Diagnostic Inc., Fort Lauderdale, USA). Glucose levels of 200 mg/dL (12 mmol/L) or above indicated diabetes development. At the end of the experiment, the animals were euthanized in a CO_2 chamber and blood and tissue samples were collected.

2.4. Tissue processing and histological analysis

Pancreas, liver, kidneys, spleen, thymus, mesenteric lymph nodes and femur were removed and fixed in buffered formalin 10% (phosphate buffer pH = 7.2) (Carlo Erba Reagents, Milano, Italy) for 24 h. The spleen was divided into two portions: one for histology and the other for lymphocyte phenotypic analysis by flow

cytometry. The paraffin blocks were cut into 5-μm-thick sections and stained with hematoxylin–eosin (HE) (Sigma–Aldrich, USA). The histophatological analysis was conducted following previously guidelines by the Society of Toxicologic Pathology (Haley et al., 2005).

2.5. Morphometric analysis of pancreatic insulitis

A quantitative evaluation of islet mononuclear infiltration was conducted using a computer-assisted image system based on a Nikon Microphot-FXA optical microscope connected via a Sony Exwave HAD video camera to a computer. To avoid inter-animal variance due to pancreatic endocrine heterogeneity, total section area of each pancreas was measured and sectioned throughout its length in five sections, sampled at regular intervals of 50 μm. Islets were traced manually and islet area was measured and expressed as a fraction of total section area.

Further, islet and inflammation areas were measured individually, as described elsewhere (Kauri et al., 2007), using the KS300 software (Carl-Zeiss, AG, Germany). The result was expressed as the percentage of islet area showing mononuclear cell infiltration, according to the formula: mononuclear infiltrate area (μm^2) × 100/islet area (μm^2). Finally, the results were categorized into the following score: 0 = intact islet; 1 = peri-insulitis (≤10% of the islet infiltrated); 2 = moderate insulitis (≤50% of the islet infiltrated); 3 = severe insulitis (≥50% of the islet infiltrated); and 4 = destructive insulitis (Santos et al., 2009). At least 20 islets per pancreas were analyzed in non-consecutive sections. To evaluate the protective effect of the extract against the loss of endocrine tissue, islets were manually traced and classified as small (300–2000 μm^2), medium (2000–10,000 μm^2), large (10,000–50,000 μm^2) and very large (>50,000 μm^2) (Kauri et al., 2007).

2.6. In situ insulin detection

Islet insulin content was evaluated by the avidin–biotin peroxidase complex (ABC) method as previously described (Pinheiro et al., 2003). Briefly, deparaffinated 5-μm tissue sections on poly-L-lysine-coated slides were treated with 0.01 M citrate buffer (pH 6.0) and heated twice, 5 min each time, in a microwave oven. To block nonspecific background staining, the sections were treated with 3% H_2O_2 in phosphate-buffered saline (PBS) for 10 min and non-fat milk for 60 min. The slides were then incubated with polyclonal guinea pig anti-insulin antibody (dilution 1:500, Dako Glostrup, Denmark) overnight and then with rabbit anti-guinea pig IgG coupled with peroxidase (Dako, Glostrup, Denmark). Chromogen color development was accomplished with 3,3′-diaminobenzidine tetrahydrochroride (Sigma). The slides were counterstained with Harris's hematoxylin. The morphometric analysis was performed using computer-assisted image system, as described in Section 2.5. Islet insulin content was established by calculating the area of immunopositive insulin relative to the islet's area, according to the formula: insulin positive area (μm^2) × 100/islet area (μm^2).

2.7. Splenic cell suspensions

Spleens were pushed against a 50 μm sterile nylon mesh (BD, New Jersey, USA) in Petri dishes containing RPMI-1640 culture medium (Cultilab, Campinas, Brazil) and the erythrocytes were lysed with an isotonic solution of ammonium chloride, potassium bicarbonate and ethylenediamine tetraacetic acid (pH 7.2) (Sigma–Aldrich, USA). Cell suspensions were washed twice (200 × *g*/10 min), and the pellet was re-suspended in RPMI-1640 culture medium that was supplemented with 10% inactivated bovine fetal serum (Cultilab, Campinas, Brazil). Cellular viability was checked using trypan blue (Hyclone, Thermo Scientific, USA) and was considered adequate for values greater than 90%. Cell suspensions were adjusted to 1×10^6 or 5×10^6 cells/mL for subsequent phenotypic analysis and cytokine evaluation, respectively.

2.8. Lymphocyte immunophenotyping by flow cytometry

Splenocyte suspensions, obtained as described in Section 2.7, were adjusted to 1×10^6 cells/mL and aliquots (100 μL) were transferred to 12 mm × 75 mm polypropylene round bottom test tubes (BD Pharmingen, San Diego, CA, USA). The samples were incubated in the dark with FITC rat anti-mouse CD4 (L3T4-clone) and PE-Cy5 rat anti-mouse CD8a (Ly-2 clone) (BD Pharmingen, San Diego, CA, USA). After 30 min, the samples were washed twice (200 × *g* during 10 min) and re-suspended in 400 μL of cold flow cytometry staining buffer (BD Pharmingen, San Diego, CA, USA). For the analysis of regulatory T cells, 100 μL of the splenocyte suspension (1×10^6 cells/mL) was incubated with FITC rat anti-mouse CD4 and PE-Cy7 rat anti-mouse CD25 following the same conditions described above. After the washing step, the pellet was submitted to a fixation/permeabilization solution (Ebioscience, San Diego, CA, USA) according to the manufacture's instruction and incubated in the dark for 30 min with PE labeled anti-mouse Foxp3 (Ebioscience, San Diego, CA, USA). The samples were washed twice (200 × *g*, 10 min) and the pellet was re-suspended in 400 μL of cold flow cytometry staining buffer. Lymphocyte subsets were determined by FACS Calibur analysis (BD, New Jersey, USA), with gating on the total lymphocyte population. For consistency, a standard calibration bead (BD Pharmingen, New Jersey, USA) was used to set the forward scatter, side scatter and PMT voltage. For each experimental sample, a corresponding isotype control was used to set gates on positive/negative cell populations. The data were represented as the percentage of positive marked cells in their respective gates.

2.9. Th1 and Th2 cytokine production

Splenocytes obtained as described in Section 2.7 were dispensed in a 48-wells flat bottomed tissue culture plate (Corning, Costar, USA) at 5×10^6 cells/mL (450 μL). The samples were stimulated with Con A (10 μg/mL) and the plates were incubated for 48 h (37 °C, 5% CO_2). The supernatants were then collected and stored at −80 °C. Th1 and Th2 cytokines were quantified using a murine Th1/Th2 cytometric bead array (CBA) kit according to the manufacturer's instructions (BD Pharmingen, USA). The FACS Calibur flow cytometer (BD Pharmingen, USA) was calibrated using setup beads, and a total of 3000 events were acquired for each sample. Individual cytokine concentrations (pg/mL) were evaluated by their fluorescence intensities and analyzed based on the standard reference curve of CELLQUEST and CBA software (BD Pharmingen, USA).

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. Data were tested for normality (Kolmogorov–Smirnov) and homogeneity (Bartlett's) and, when necessary, submitted to appropriate transformations. Body weight, water and food intake, organ weight, hematological parameters, immunophenotyping, cytotoxicity data and cytokine concentration were compared by ANOVA. When ANOVA results were significant ($p < 0.05$), the comparison was followed by the Tukey test. For the non-parametric data, the Kruskal–Wallis test was applied. Glycemic levels were analyzed by Friedman test.

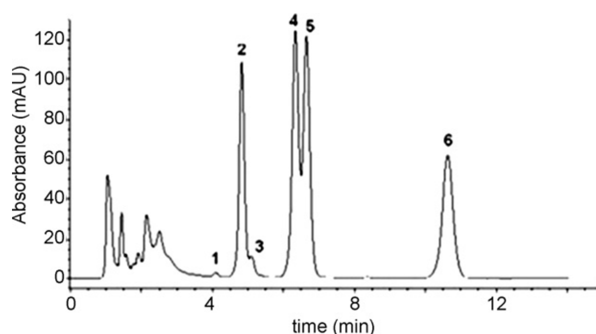

Fig. 2. HPLC–UV pentacyclic oxindole alkaloid profile (Laus and Keplinger, 1994 conditions) of the studied *Uncaria tomentosa* alkaloidal fraction. (1) Speciophylline; (2) mitraphylline; (3) uncarine F; (4) pteropodine; (5) isomitraphylline; (6) isopteropodine. For extract quantification details see Reis et al. (2008).

3. Results

3.1. Pentacyclic oxindole alkaloid profile and content

The previous HPLC analysis (Pereira et al., 2008; Reis et al., 2008) of this bioactive aqueous–ethanol extract of *Uncaria tomentosa* indicated the presence of the six pentacycle oxindole alkaloid that were considered to be chemical markers of the species: speciophylline, mitraphylline, uncarine F, pteropodine, iso-mitraphylline and isopteropodine (Fig. 2). The total alkaloid content, usually the marker for assessing the pharmaceutical quality of the cat's claw extracts, was 29.1 mg/g (±1%) which is in agreement with commercially acceptable plant materials.

3.2. Effect of *Uncaria tomentosa* on the incidence of diabetes

As expected, inoculation with MLDS was associated with a successful diabetes induction, characterized by high glucose concentrations. Mice injected with MLDS presented glycemic levels significantly higher at experimental days 12 and 21. Treatment with 80, 100 and 400 mg/kg of the *Uncaria tomentosa* extract determined a significant reduction in the glycemic levels at the end of the experimental period (Fig. 3a).

All tested doses (10–400 mg/kg) promoted a delay in diabetes incidence during the experimental period and, furthermore, the highest *Uncaria tomentosa* dose completely abrogated diabetes incidence at day 12, preventing the raise of glycemic levels above 200 mg/dL in most of the animals at day 21 (Fig. 3b).

3.3. Histopathology and morphometric analysis of insulitis and insulin content

The histological analysis of spleen, lymph nodes, kidneys, thymus, femur and liver did not reveal significant structural differences among the untreated and treated groups.

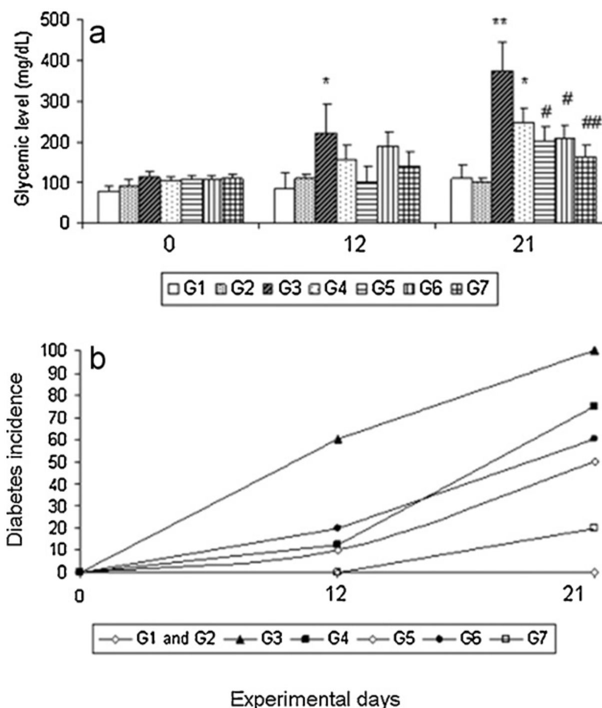

Fig. 3. Effect of *Uncaria tomentosa* extract on glycemic levels and diabetes incidence. (a) *Uncaria tomentosa* extract determined a significant reduction of glycemic levels (mean ± SD). *,**Significantly higher than the observed in G1 and G2 groups; $p < 0.05$ and $p < 0.001$, respectively. #,##Significantly lower than the observed in G3 group; $p < 0.01$ and $p < 0.001$, respectively. (b) *Uncaria tomentosa* extract delayed diabetes incidence. Values are expressed as percentage of diabetic animals. G1 and G2: negative control and *Uncaria tomentosa* at 400 mg/kg, respectively; G3: MLDS control; G4–G7: MLDS + *Uncaria tomentosa* at 10, 50, 100 and 400 mg/kg, respectively.

As expected, animals injected with MLDS presented a clear pattern of insulitis, with the predominance of moderate (grade 2) and invasive (grade 3) insulitis. At the highest experimental dose (400 mg/kg), *Uncaria tomentosa* extract produced a significant protection against mononuclear infiltration, characterized by the higher percentage of intact islets (grade 0) and a concomitantly reduction in the percentage of moderate, severe and destructive insulitis (Fig. 4a). These effects were accompanied by preventive reduction in islet size determined by MLDS, resulting in a predominance of medium and large islets (Table 1). Reduced frequency of apoptotic bodies, cytoplasmatic vacuolization and eosinophilia were also observed, reinforcing a protective effect against destructive insulitis (data not showed).

As expected, insulin production by β-cells was clearly reduced in animals injected with MLDS (Fig. 4b). However, the *Uncaria tomentosa* treatment, particularly at 400 mg/kg, promoted the highest preservation in the insulin content *in situ* (Fig. 4c–e).

Table 1
Islet size[a] in C57BL/6 mice injected with MLDS and orally treated with the aqueous–ethanol extract of *Uncaria tomentosa* during 21 days.

	Small (<2000 μm^2)	Medium (<10,000 μm^2)	Large (<50,000 μm^2)	Very large (>50,000 μm^2)
G1 – negative control	0	15	75	10
G2 – UT 400 mg/kg	0	20	66	14
G3 – MLDS control	42	48	10	0
G4 – MLDS + UT 10 mg/kg	25	61	14	0
G5 – MLDS + UT 50 mg/kg	16	67	17	0
G6 – MLDS + UT 100 mg/kg*	0	40	48	12
G7 – MLDS + UT 400 mg/kg*	0	15	71	14

[a] Islet size was measured using a computer-assisted image system and expressed as relative values (%) for each experimental group.
* Groups G6 and G7 presented islet size significantly higher than the observed in G3 group ($p < 0.001$–chi-square test).

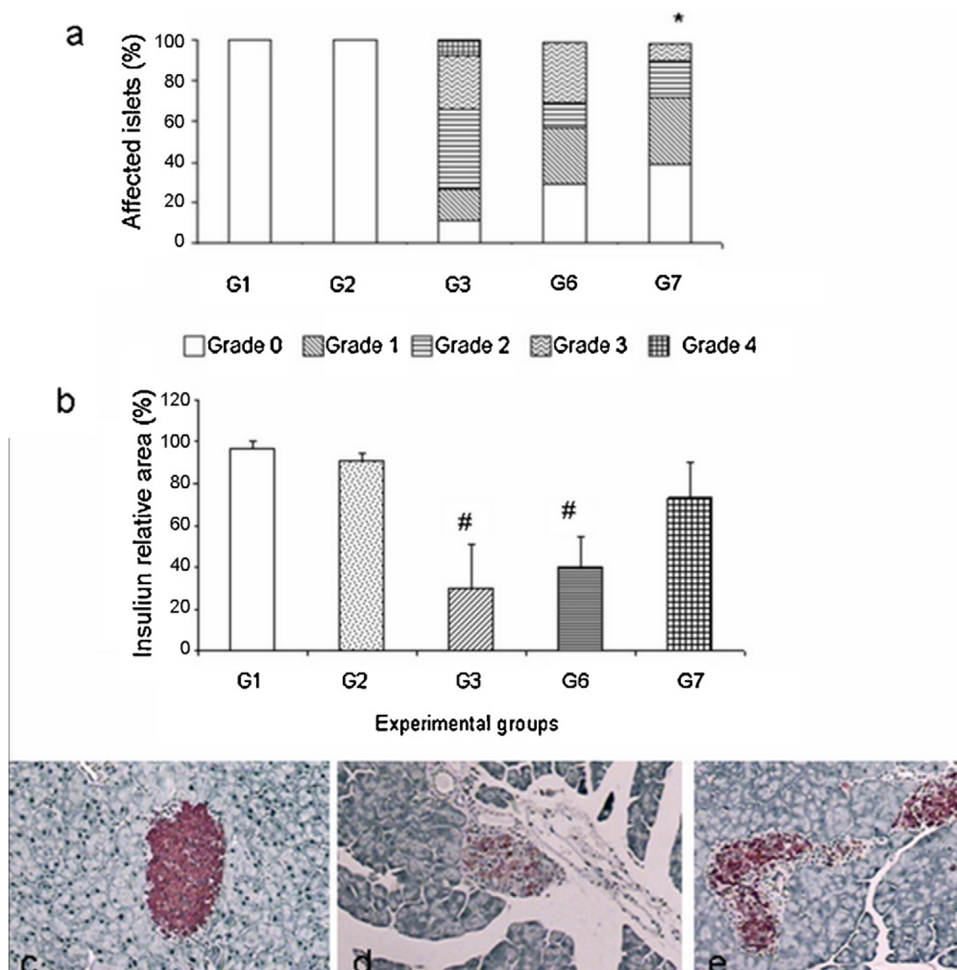

Fig. 4. Protective effect of *Uncaria tomentosa* extract on the incidence of insulitis (a) and insulin production (b). *Higher number of intact islets (Grade 0) when compared to G3 group ($p < 0.05$, chi-square test). #Significantly different from G1 and G2 control groups ($p < 0.001$). Figures (c)–(e) indicated *in situ* insulin content. (c) G1 control group, (d) G3 MLDS group and (e) G7 *Uncaria tomentosa* extract 400 mg/kg group, respectively. G1 and G2: negative control and *Uncaria tomentosa* extract 400 mg/kg, respectively; G3: MLDS control; G6 and G7: MLDS + *Uncaria tomentosa* extract at 100 and 400 mg/kg, respectively.

3.4. Phenotypic analysis of splenic lymphocyte subsets

Animals injected with MLDS presented a reduced number of $CD4^+$ T cells. However, treatment with *Uncaria tomentosa* extract at 100 and 400 mg/kg determined a significant increase in the number of $CD4^+$ T cells. The phenotypic analysis did not reveal significant difference regarding the percentage of $CD8^+$ T cells among the groups (Table 2). Injection of MLDS also reduced the percentage of $CD4^+CD25^+Foxp3^+$ Treg lymphocytes. However, treatment with *Uncaria tomentosa* extract at 100 and 400 mg/kg resulted in Treg cell values similar to those observed in the control groups (Table 2).

3.5. Effect of Uncaria tomentosa extract on Th1/Th2 cytokine production

Animals injected with MLDS produced a Th1 cytokine profile, characterized by higher IFN-γ levels and a non-significant decrease of IL-4 and IL-5 levels, in comparison to the control groups. On the other hand, treatment with *Uncaria tomentosa* prevented this

Table 2
Phenotypic analysis[a] of splenic lymphocyte subsets in C57BL/6 mice injected with MLDS and orally-treated with the aqueous–ethanolic extract of *Uncaria tomentosa* during 21 days.

	$CD4^+$ cells	$CD8^+$ cells	$CD4^+CD25^+Foxp3^+$ Tregs
G1 – negative control	24.22 ± 1.1	15.2 ± 2.5	3.2 ± 0.3
G2 – UT 400 mg/kg	23.0 ± 1.1	14.5 ± 0.9	3.0 ± 0.6
G3 – MLDS control	18.82 ± 0.6*	12.5 ± 0.4	2.3 ± 0.4*
G4 – MLDS + UT 100 mg/kg	24.26 ± 1.6	15.4 ± 3.5	2.7 ± 0.6
G5 – MLDS + UT 400 mg/kg	23.2 ± 1.5	14.9 ± 0.6	2.9 ± 0.5

[a] Values are expressed as percentage of lymphocyte subsets (mean ± SD), 10,000 cells/animals were counted.
* Significantly different from G1 group, $p < 0.05$.

Th1 polarization and also determined a significant increase in IL-4 production (Fig. 5).

4. Discussion

Immune-mediated diabetes is a chronic disorder associated, in genetically susceptible individuals, with generation and activation of autoreactive T cells (Chentoufi et al., 2008). Experimental immunotherapies for the disease involve a broad spectrum of immunoregulatory strategies, including deletion of lymphocyte subsets or the use of substances that induce immune tolerance through T regulatory (Treg) cells activation (Kohm et al., 2005; Chung et al., 2007). Some of these approaches have shown efficacy in clinical trials, but risks such as abnormal cytokine release and/or reactivation of latent viruses render difficult its adoption (Luo et al., 2010). In this context, herbal immunomodulators could be considered potential adjuvant therapies. *Uncaria tomentosa* is being largely used as antitumoral, antioxidant and immunomodulatory agents (Allen-Hall et al., 2010; Dreifuss et al., 2010). Our study is the first to demonstrate that an aqueous–ethanolic *Uncaria tomentosa* extract was able to prevent the full evolution of experimental diabetes. Immune-mediated diabetes was induced by injecting C57BL/6 mice with multiple low streptozotocin doses (MLDS diabetes). This procedure has been reported as capable to initiate a minor chemical destruction of β cells and shedding of islet's self-antigens. As a consequence, immune cells respond to β-cell self-antigens and the progression of hyperglycemia and insulitis evolve during a 2-week period (Cvjetićanin et al., 2010). This MLDS model has been largely employed to study the immunological mechanisms underlying diabetes, including its immunopathogenesis and also therapeutic strategies (Karabatas et al., 2005; Kanitkar et al., 2008).

Many parameters sustained the protective effect of *Uncaria tomentosa* against the progression of diabetes in the MLDS model. We initially observed that all tested doses were able to decrease glycemic levels. This effect was already observed at experimental day 12 but was more pronounced at day 21. Moreover, this activity showed to be dose-dependent; the highest concentration being the most efficient one. Notably, the highest *Uncaria tomentosa* dose (400 mg/kg) was able to reduce the diabetes incidence to 15%, whereas the STZ non-treated group displayed 100% of diabetes incidence. Similar hypoglycemic activity has been attributed to other plant-derived natural products, such as *Cassia auriculata* and *Bauhinia forficata* (Gupta et al., 2009; Da Cunha et al., 2010). This protective potential has been associated with diverse mechanisms such as restoration of pancreatic function, increase in insulin release and inhibition of glucose intestinal absorption (Jia et al., 2003). However, only a few of these evaluations were performed using experimental models of immune-mediated diabetes (Cvjetićanin et al., 2010; Sonawane et al., 2010).

We believe that the protective effect of *Uncaria tomentosa* on MLDS-induced diabetes was, at least partially, due to a diminished islet inflammatory process. Control mice injected with STZ presented a pattern of severe insulitis, characterized by the predominance of moderate to severe insulitis. On the other hand, treatment with *Uncaria tomentosa* extract changed this pattern by increasing the percentage of intact islets. A reduced frequency of parameters usually linked to islet disfunction, such as apoptotic bodies, vacuolization and eosinophilia, was also observed after *Uncaria tomentosa* treatment. Interestingly, the extract also preserved much of the ability of the islet to produce insulin, as demonstrated by the *in situ* immunohistochemistry analysis.

The tight association between pancreatic infiltration of the major orchestrators of β-cell damage, such as auto-reactive $CD4^+$ and $CD8^+$ lymphocytes, and destruction of insulin-secreting β-cell was recently reported (Wesley et al., 2010). Immune-mediated diabetes has been classically described as a predominant Th1 mediated disease, characterized by an elevated production of IFN-γ

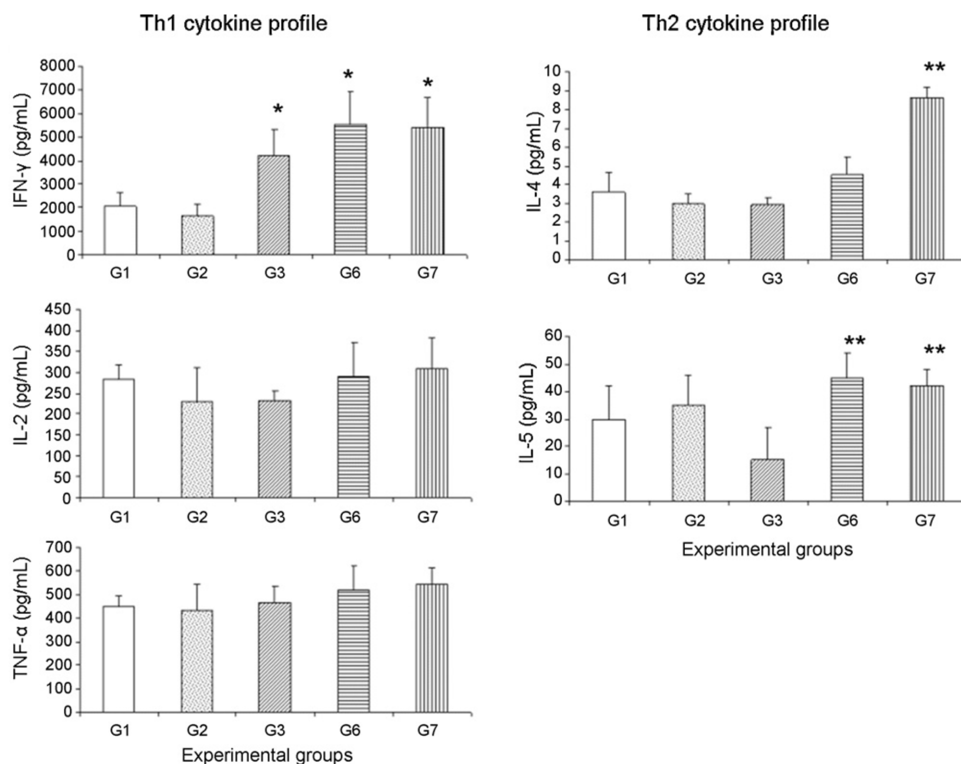

Fig. 5. Effect of *Uncaria tomentosa* extract on the mitogen-induced Th1 and Th2 cytokine production. *Significantly different from G1 group, $p < 0.05$; **Significantly different from G3 group, $p < 0.05$. G1 and G2: negative control and *Uncaria tomentosa* 400 mg/kg, respectively; G3: MLDS control; G6 and G7: MLDS + *Uncaria tomentosa* extract at 100 and 400 mg/kg, respectively.

and IL-2 in both human patients and experimental models (Csorba et al., 2010). In this context, it has been assumed that a naturally predominant or a protocol-induced Th2 profile might avoid or ameliorate diabetes manifestations (Muller et al., 2002; Christen and von Herrath, 2004). In this investigation we also confirmed this Th1 polarization in STZ injected mice by evaluating cytokine production in spleen cells stimulated with ConA. Cell cultures from diabetic mice produced higher IFN-γ levels than normal animals. No differences were observed in IL-4 levels. Treatment with *Uncaria tomentosa* did not modify the high IFN-γ levels, but the highest extract dose significantly increased both IL-4 and IL-5 levels. These results suggested that the observed protective effect could be due, at least partially, to a Th2 polarization. In fact, previous studies have demonstrated the potential of herbal extracts to modulate the incidence and progression of immune-mediated diabetes through the dynamic modulation of cytokines toward a Th2 profile (Li et al., 2007; Chen et al., 2008).

As $CD4^+CD25^+$Foxp3 regulatory T cells (Treg cells) are essential to maintain self-tolerance (Wan, 2010) and have also been a promising target in autoimmunity therapy (Hsieh and Bautista, 2010), their possible contribution to the protection determined by *Uncaria tomentosa* was evaluated. Our data showed that STZ-diabetic mice presented a significant reduction in the percentage of $CD4^+CD25^+$Foxp3 Treg cells in the spleen. Treatment with the *Uncaria tomentosa* extract restored the number of splenic Treg cells to values similar to those observed in healthy animals. Our interpretation of this data is based on evidences that Treg cells dislocate from secondary lymphoid organs to the target sites to control the inflammatory process (Kim, 2006). We could first hypothesize that natural Treg cells that are usually present in the spleen, relocated to the pancreas during diabetes to control insulitis. However, they were not effective due to their small number or low activation status. In this context, we could think that *Uncaria tomentosa* treatment could modulate Treg cell subset by increasing their number and activation stage. This could partially control diabetes and restore Treg cell number in the spleen. This possibility is, at some extension, supported by literature, once the Treg cells have indeed been reported to exert control of insulitis in the MLDS model (Zdravkovic et al., 2009). In addition, it was demonstrated in NOD mice that peripheral Treg cells were able to prevent diabetes by initially homing to the pancreas (Lepault and Gagnerault, 2000).

It is important to highlight that this treatment, even at the highest extract doses, was not associated with any signs of systemic or organ-specific toxicity. These findings are partially supported by a previous work that demonstrated a LD50 higher than 8 g/kg for the low-alkaloid content *Uncaria tomentosa* aqueous extract (Sheng et al., 2000). Taken together, our results indicate that *Uncaria tomentosa* was able to protect mice against immune-mediated diabetes. The immunological assays indicated that this protective ability could be determined by a Th2 polarization, an expansion of Foxp3+ Treg cells, or both mechanisms. As this protection was devoid of any relevant associated deleterious effect, we believe that this extract, or more purified derivatives, are worthwhile to be considered in human diabetes treatment and, therefore, should be extensively studied.

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