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Anonna muricata L. (soursop) seed oil improves type 1 diabetes parameters *in vivo* and *in vitro*

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ABSTRACT

Annona muricata have many properties reported as antidiabetic, antitumor and antioxidant effects. However, studies on the use of fruit seeds are limited. In this study, we identified the immunomodulatory potential of *A. muricata* seed oil (AmSO) in streptozotocin (STZ)-induced *in vivo* and *in vitro* experimental model of T1D and whole blood cell culture from diabetic patients. AmSO cytotoxicity was evaluated by MTT-tetrazolium and resazurin. AmSO was orally administered to BALB/c mice for 48 days and were divided into groups: control, STZ (diabetic), STZ–AmSO (diabetic, treated with 1.0 mg/kg AmSO), and AmSO groups (nondiabetic, treated with 1.0 mg/kg AmSO). T1D was induced with intraperitoneal administration of STZ (3 × 100 mg/kg). Biochemistry and histopathological analysis and area of pancreatic islets were evaluated. IL-10, IL-4, IL-17 production in spleen cell culture from diabetic mice exposed to AmSO and IFN-γ and IL-10 levels in whole blood cell culture from diabetic patients exposed to AmSO was determined by ELISA. AmSO showed antihyperglycemic effect, preserved the area of pancreatic islets, preserved liver tissue, increased IL-4 and IL-10 levels in spleen cell culture, and decreased IFN-γ level in whole blood cell culture. AmSO demonstrated immunomodulatory effect and therapeutic potential for the treatment and/or prevention of clinical T1D.

1. Introduction

Type 1 diabetes mellitus (T1D) is a metabolic disorder that causes destruction of β cells from the islets of Langerhans in the pancreas and usually leads to an absolute insulin deficiency [1]. Several factors are implicated in the onset and progression of T1D, including genetic, immunological, and environmental mechanisms [2]. Approximately 30% of all newborn children have the genetic risk for T1D [3] and a smaller percentage of these children progress to the clinical disease. Additional factors are also involved in T1D pathogenesis, such as viral infections, intestinal inflammation, and nutritional factors, which are required to trigger the disease in genetically predisposed individuals [2].

This disease is manifested when over 80% of β cells are destroyed and insulin production is insufficient to meet the metabolic demand of the body [4]. Among the immunological components involved in T1D pathogenesis are T cells ($CD4^+$ and $CD8^+$); B cells [5,6]; antigen-presenting cells; specific autoantibodies, such as antipeptides of β cells, anti-insulin, anti-GAD (GAD65), and antitirosina phosphatase (IA-2 and Ia-2b) [1]; and pro-inflammatory cytokines, especially Th1, such as IL-β, TNF, IFN-γ, Th17, and IL-17 [7,8] in addition to a higher expression

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of both histocompatibility complex molecules and costimulatory molecules [6].

After the initiation of autoantibody production, the risk for T1D increases by 75% within 10 years and manifestation of the clinical disease is likely within 20 years [9]. Thus, strategies aimed at preventing pathogenesis and progression of T1D, such as immune deviation from Th1 to Th2 and increased regulatory cytokines, are extremely important. New drugs or nutraceuticals obtained from plants with anti-inflammatory and immunomodulatory properties, with low side effects, are advantageous strategies for controlling this chronic disease.

Anonna muricata L. (soursop) has several biological properties, such as anti-inflammatory [10], antidiabetic, antioxidant [11], and hypoglycemic [12] properties.

Besides the action of bioactive constituents in medicinal plants, some studies have indicated that their biological effects are a result of the metabolic interaction with fatty acids which promote diabetes control [13,14]. Insulin secretion may be mediated by activated metabolic processes when fatty acids reach the cytoplasm and/or bind to GPR40 receptors [15]. Other models of acute exposure of β cells to fatty acids support the hypothesis of amplification of insulin secretion by modulating glucose metabolism. β cells can respond to the action of fatty acids at the transcription and transduction levels, promoting its function and survival [16].

The main fatty acids of the soursop seeds are oleic acid (40.94%), linoleic (33.02%), and palmitic acid (18.98%) [17]. Oleic and linoleic fatty acids are reported to have antidiabetic and cytoprotective effects [16,18–20]. Thus, we hypothesized that soursop seeds, especially seed oil, have the potential to improve T1D progression in mice.

Thus, this study aimed to evaluate the immunomodulatory activity of *A. muricata* seed oil in streptozotocin (STZ)-induced *in vivo* and *in vitro* experimental model of T1D and in whole blood cell culture from patients with T1D.

2. Methods

2.1. A. muricata seed oil (AmSO) extraction

Soursop seeds were kindly provided by an industry of fruit pulp located in Ilhéus (Lat. 14° 47′20″S and Long. 39° 02′58″W), Southern Bahia, Brazil, and were lyophilized to facilitate grinding and better extraction of ethereal components. Oil was extracted and adapted according to the Bligh–Dyer methodology [21] using methanol and chloroform as solvents. The decanted phase in the extraction process was collected and concentrated on a rota-evaporator equipment at 10G under vacuum at 50–55 °C for 45 min and was then subjected to a final drying by nitrogen gas.

2.2. T1D experimental model

2.2.1. Animals and ethical considerations

BALB/c mice aged 8–10 weeks were used in this study. The animals were housed at 22 °C and received water and food *ad libitum* until the time of the experiment. Animal procedures were performed in accordance with the recommendations of the Ethics Committee on Animal Use from Oswaldo Cruz Foundation (CEUA-CPqGM 011/2014).

2.2.2. Effect of AmSO on spleen cells in vitro

AmSO was dissolved in RPMI (Gibco) with 0.5% DMSO (dimethyl sulfoxide, Sigma) in RPMI followed by filtration (0.2 μm). AmSO contained waste precipitates that were retained after filtration. Thus, a quantity of 40% of AmSO weight was added to correct the average percentage weight loss during filtration. Spleen cell suspension (200 μL) collected aseptically and enriched with 5% FBS and antibiotics was added in a 96-well microplate (5×10^6 cells/well) in the presence or absence of AmSO solution in serial dilution (1000 to 7.81 μg/mL). DMSO (50%) was used as a positive control.

2.2.2.1. Cell viability by MTT-tetrazolium. After 72 h of incubation at 37 °C and 5% CO_2, 20 μL of MTT-tetrazolium (Sigma-Aldrich, 5 mg/mL) was added to the spleen cell culture. After 4 h, plates were centrifuged at 1008G/10 min. Supernatant (170 μL) was carefully discarded and 100 μL of DMSO was added into each well. After 10 min of reaction, plates were read at 570 nm (adapted from Mosmann [22]). Absorbance of AmSO unexposed control cells were considered to calculate 100% of cell viability.

2.2.2.2. Cell viability by sodium resazurin. After 72 h of incubation at 37 °C and 5% CO_2, 15 μL of resazurin solution (Sigma-Aldrich, 0.312 mg/mL) was added to the spleen cell culture. The plates were reincubated overnight and subjected to reading at 570 and 595 nm. The difference of resazurin reduction of control cells was calculated according to the manufacturer's recommendations and regarded as 100% cell viability (adapted from Rampersad [23]).

2.2.3. T1D protocol in vivo

The total number of days to perform DM1 protocol was 48 days (D48) (adapted Cerqueira-Lima et al. [24]). Cerqueira-Lima et al. [24] performed an experimental model of diabetes with STZ dose at 100 mg/kg for 3 consecutive days, intraperitoneally, in strains of mice BALB/c male. In this model, the atrophy of the islets of the STZ-diabetic animals was observed compared with controls.

The experimental design of STZ-induced T1D can be found in the supplemental material (S1). BALB/c mice (7–8 weeks) were divided into four groups: 1) Ctrl, control group (received saline orally and three consecutives 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) STZ–AmSO group, received oral AmSO (1.0 mL/kg) and three i.p. injections of STZ (100 mg/kg); and 4) AmSO group, received oral AmSO (1.0 mL/kg) and three i.p. injections of citrate buffer, pH 4.5). AmSO was orally administered on D0–D47. The dose of 1.0 mL/kg was applied based in a pilot study testing for toxicity in mice conducted by our research group. On D33–D35, T1D was induced by i.p. administration of STZ. After 5 days of STZ injection (D40), the effectiveness of diabetes induction was evaluated by measuring blood glucose level (> 200 mg/dL). STZ injections were administered to animals with 8 h of fasting. Fasting was also applied before measure blood glucose level. On D48, the animals were euthanized (150 mg/kg thiopental + 10 mg/kg lidocaine) and samples were collected for analysis.

2.2.3.1. Biochemical and histopathological analysis. Fasting glycemia was assessed on D0 and D47 from blood obtained from the tail vein using an electrical feeder (Accu-Chek Active, Roche). Insulin level was measured with ELISA (Ultrasensitive Mouse Insulin ELISA, Mercodia, Uppsala, Sweden) in serum from control and diabetic mice.

Biopsies from the liver and pancreatic tissues of the mice were harvested. Samples were paraffin embedded twice after fixing with 10% formalin using a microtome, and paraffin blocks were cut into serial 3 μM sections. Next, slides were immersed in hematoxylin-eosin (HE) for staining. Additionally, the Masson's trichrome and periodic acid shift (PAS) staining was performed on the liver samples to evaluate any diabetes-induced histological damages. Histopathological changes were evaluated by two different pathologists in a blinded manner. Reading was performed with light microscopy (Nikon Labophot) at 100 × and 400 × . The pancreatic islet area was measured using Image G software. Alanine transaminase serum (ALT) and serum creatinine levels were evaluated and performed in semiautomatic equipment Bioplus 2000 using commercial kits (Reagents Doles®, Brazil) according to the manufacturer's recommendations.

2.2.3.2. Cytokines assay in vitro. After euthanasia, spleen cells from the STZ group were distributed into culture plates in the absence and/or presence of AmSO at concentrations 31, 62.5, and 125 μg/mL. AmSO