UNIVERSIDADE ESTADUAL PAULISTA
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Avaliação dos mecanismos de ação envolvidos na atividade anti-diabetogênica do extrato hidroetanólico da folha da *Smallanthus sonchifolius* (yacon) em ratos com Diabetes mellitus experimental

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutor(a) em Patologia.

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Resumo

Resumo

O diabetes mellitus tipo 1 (DM1) ocorre pela destruição das células β pancreáticas, levando a deficiência de secreção e síntese de insulina e perda de controle glicêmico, resultando em maior uso de ácidos graxos como fonte de energia. A utilização excessiva de ácidos graxos leva ao estresse oxidativo nos cardiomiócitos que está relacionado à cardiomiopatia diabética. Além disso, a superprodução de espécies reativas de oxigênio e a diminuição da defesa antioxidante têm impacto negativo na função muscular, como comprometimento do crescimento muscular e força e capacidade metabólica alterada. Uma vez que a cardiomiopatia diabética e a resposta adaptativa do músculo esquelético estão estreitamente relacionadas ao estresse oxidativo, o tratamento com folhas de Yacon pode ser útil para diminuir/prevenir a progressão dessas complicações diabéticas. O objetivo deste estudo foi investigar o efeito protetor do extrato das folhas de Yacon (YLE) sobre a disfunção metabólica, cardiomiopatia e resposta adaptativa do músculo esquelético em ratos diabéticos. Ratos Wistar machos (10/grupo) foram alocados da seguinte forma: C, controles; C + Y, controles tratados com YLE; DM, controles diabéticos; e DM + Y, ratos diabéticos tratados com YLE. O T1DM foi induzido pela administração de estreptozotocina (40mg/kg/peso corporal). Os grupos tratados receberam 100 mg/kg de YLE por dia (via gavagem) por 30 dias. O grupo tratado apresentou melhora na disfunção metabólica na condição diabética (DM vs. DM + Y), melhorando a lesão das ilhotas pancreáticas, liberação de insulina e a hiperglicemia, aumentando a atividade das enzimas antioxidantes em tecidos cardíacos e no músculos sóleo, além de diminuir a fibrose e desorganização celular no tecido cardíaco. Os benefícios aparentes do tratamento com YLE parecem ser mediados pela melhora da disfunção metabólica e do estresse oxidativo nos tecidos cardíaco e muscular. Portanto, explorar potenciais efeitos terapêuticos de plantas e fitoterápicos pode contribuir para detectar novos alvos e tratamentos. Nossas descobertas, mesmo que em

modelo básico, fornecem informações que podem orientar estudos futuros com o objetivo de elucidar novas alternativas terapêuticas para complicações diabéticas.

Abstract

Abstract

Type 1 diabetes mellitus (T1DM) occurs by destruction of pancreatic β-cells leading to insulin deficiency and loss of glycemic control, resulting in increased fatty acids (FA) utilization as energy source. FA over-utilization leads to oxidative stress in the cardiomyocytes which is related to diabetic cardiomyopathy. The overproduction of reactive oxygen species and decreased antioxidant defense have a negative impact on muscle function, such as impairment of muscle growth and strength and altered metabolic capacity. Since diabetic cardiomyopathy and the adaptive response of skeletal muscle are closely related to oxidative stress, treatment with Yacon leaves may be useful to decrease/prevent the progression of these diabetic complications. The aim of this study was to investigate the protective effect of Yacon leaves extract (YLE) on dysmetabolism, cardiomyopathy and soleus muscle adaptive response to stress in diabetic rats. The rats (10/group) were allocated as follows: C, controls; C+Y, controls treated with YLE; DM, diabetic controls; and DM+Y, diabetic rats treated with YLE. T1DM was induced by administration of streptozotocin (40mg/kg/body weight). treated groups received 100 mg/kg YLE daily via gavage for 30 d. The YLE group improves dysmetabolism in diabetic condition (DM vs. DM+Y), by ameliorating the pancreatic islet injury, insulin release and hyperglycemia, as well as increasing the activity of the antioxidant enzymes in cardiac and skeletal muscle and decreasing the fibrosis and cellular disorganization in cardiac tissue. The apparent benefits of YLE seem to be mediated by ameliorating dysmetabolism and oxidative stress in pancreas, heart and soleus tissue. Therefore, exploring potential therapeutic effects of plants and herbal medicines can help detect new targets and treatments. Our findings, even in a basic model, provide information that may guide future studies to elucidate new therapeutic alternatives for diabetic complications.

Sumário

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Capítulo I

Introdução

Capítulo I – Introdução

Diabetes mellitus: Aspectos Gerais e Prevalência

Diabetes mellitus é uma doença crônica complexa, crônica e que requer atenção médica continua (1). Trata-se de uma desordem metabólica caracterizada pela secreção e/ou ação da insulina endógena deficiente, acompanhada por hiperglicemia crônica e persistente. Vários fatores estão envolvidos nesta patologia, sendo que os nutricionais e a autoimunidade são os principais preditores da patogênese (2).

A prevalência e o número de casos de diabetes têm aumentando expressivamente nas últimas décadas (3). No mundo, estima-se que 422 milhões de adultos viviam com diabetes em 2014, comparados a 180 milhões em 1980. A prevalência global basicamente dobrou desde 1980, passando de 4,7% para 8,5% da população adulta (3). Contudo, não existem dados globais separados sobre a prevalência do diabetes tipo 1 (DMT1) e tipo 2 (DMT2).

Ambos os tipos (DMT1 e DMT2) são doenças heterogêneas, e a classificação é importante para determinação da terapia adequada. Contudo, o paradigma tradicional de que o DMT2 ocorre apenas em adultos e que o DMT1 ocorre apenas em crianças não é mais válido, uma vez que as duas formas da doença ocorrem em ambos grupos etários (4). O início e progressão do DMT1 pode ser mais variada em adultos e não necessariamente apresenta os mesmos sintomas clínicos vistos em crianças. Apesar das dificuldades da distinção de ambos os tipos em diferentes grupos etários, o diagnóstico se torna evidente ao longo do tempo.

Resumidamente, é considerado grave problema de saúde pública, uma vez que está associada a complicações que comprometem a produtividade, qualidade de vida e sobrevida dos indivíduos afetados, além de envolver altos custos no seu tratamento (5).

Diabetes mellitus tipo 1: Fisiopatologia – Alterações Metabólicas e Estresse Oxidativo

O Diabetes mellitus do tipo 1 é uma doença autoimune órgão-específica caracterizada pela destruição seletiva de células-beta pancreáticas produtoras de insulina. Alterações drásticas ocorrem no metabolismo de pessoas com DMT1 durante a privação de insulina e, consequentemente, aumento da glicose plasmática. Sob essas condições, a quebra de proteínas estruturais e a lipólise estão aumentadas, levando a perda de peso e aumento dos lipídios circulantes (6). O processo da lipólise envolve a hidrólise de triacilglicerol estocados no tecido adiposo à glicerol e ácidos graxos. Como resultado, os ácidos graxos são liberados para a circulação, elevando sua disponibilidade como fonte energética resultando em sua excessiva oxidação (7). Devido as alterações metabólicas, com diminuição expressiva da síntese e secreção de insulina e, consequentemente diminuição da captação de glicose, os ácidos graxos são utilizados como fonte energética majoritária, principalmente pelo tecido cardíaco (7). Contudo, este processo ocorre também no músculo esquelético de indivíduos portadores de DMT1, levando ao depósito ectópico de gordura (8), levando ao processo de lipotoxicidade nesses tecidos.

Concomitantemente, a desregulação do metabolismo está diretamente relacionada ao aumento da produção de espécies reativas do oxigênio, favorecendo o estresse oxidativo, que leva à danos ao DNA, proteínas e lipídios, assim como ativação de vias metabólicas estresse-sensíveis em ambos os tecidos em questão (cardíaco e músculo esquelético) (9,10), causando danos às estruturas celulares com resultantes deficiências funcionais. Estudos experimentais e clínicos revelaram que a hiperglicemia estabelecida no diabetes está associada ao estabelecimento do estresse oxidativo (11) desde que o excesso de glicose circulante é oxidada excedendo a capacidade natural do sistema antioxidante, desempenhando papel importante na progressão e complicações do DM (7,12,13). O aumento de marcadores de estresse oxidativo e

inflamatórios já foram identificados em pacientes com DMT1. Estes fatores apresentam impacto negativo na saúde do músculo esquelético (14) e do tecido cardíaco (15), levando à perda de massa muscular, estresse metabólico e cardiomiopatia diabética (16).

Portanto, estratégias terapêuticas que levem a homeostase glicêmica e reduzam o shift metabólico gerado na condição diabética, favorecendo a utilização de glicose e diminuindo a oxidação de ácidos graxos levando, consequentemente, a melhor do metabolismo energético e diminuição do estresse oxidativo tornam-se de relevância significativa. A utilização de agentes antidiabéticos com capacidade antioxidante é altamente recomendada. Plantas medicinais já são vastamente utilizadas como terapia alternativa e complementar para a prevenção e/ou tratamento de diversas doenças. Nos últimos anos, grande atenção tem sido dada a compostos naturais, devido as suas características e atividades farmacológicas e nutricionais (17).

Yacon (*Smallanthus sonchifolius*): Origem, Características Botânicas, Generalidades, Composição Química e Atividades Biológicas

O Yacon, *Smallanthus sonchifolius* (Poepp.) H. Rob., é uma planta perene e herbácea nativa da Cordilheira dos Andes (18), com expansão botânica nas regiões da Venezuela para o Noroeste da Argentina, oeste da Europa, Nova Zelândia, Japão, EUA, República Checa e Estado de São Paulo (19), conhecida popularmente como batata-yacon e batata-do-diabético no Brasil.

A espécie pertence à família Asteraceae e foi descrita pela primeira vez como *Polymnia sonchifolia* Poepp. por Eduard Friedrich Poeppig em 1845. Uma perspectiva diferente foi adotada por Harold Ernest Robinson, que restabeleceu o gênero *Smallanthus* e separou as espécies antes consideradas *Polymnia* em dois gêneros diferentes - *Smallanthus* e e *Polymnia* - na mais recente revisão do gênero, publicada em 1978. Apresenta ainda uma

sinonímia botânica, *Polymnia edulis* Wedd., publicada em 1857 (20). Trata-se de um subarbusto perene, decíduo, ereto, pouco ramificado, com raiz tuberosa e curtos rizomas, de caule geralmente arroxeado e hirsuto-pubescente, de 50-100 cm de atura. As folhas têm pecíolo alado, de lâmina cartácea e esparso-pubescente na face inferior que tem coloração quase branca, de 10-25 cm de comprimento (18). Adapta-se a uma grande variedade de solos, mas se desenvolve melhor em solos ricos, moderadamente profundos a profundos, bem estruturados e bem drenados. Cresce muito bem nos solos lateríticos corrigidos com calcário dolomitico no estado de São Paulo, Brasil e pode tolerar uma ampla gama de pH, de ácido a fraco-alcalino (21,22). No estudo anatômico desenvolvido por Machado et al., (2004) (23) ficou evidenciado claramente que a organização do sistema vascular do órgão subterrâneo do Yacon é típica de raiz, embora se encontrem referências a tubérculo e rizoma na literatura, as quais não são adequadas, uma vez que estes são órgãos subterrâneos de natureza caulinar. Segundo os autores, o aumento de espessura das raízes tuberosas é resultante da proliferação de tecido parenquimático, o qual pode acumular açúcares e pigmentos como o amarelo-laranja os quais podem ser influenciados pelo genótipo da planta (24).

Seu cultivo é produtivo com rendimentos de matéria seca de raízes em solos de fertilidade moderada superior a 10 t / ha em 6-8 meses de cultivo. As raízes de casca escura variam de esférico a oblongo e pesam de 100 g a 1 kg. É uma cultura de raízes não amiláceas consumida como fruta em sistemas alimentares tradicionais Andinos (21,24). O cultivo do Yacon possui finalidades alimentícias provenientes das suas folhas por meio do seu consumo na forma de chás, extratos brutos e orgânicos, e das suas raízes ingeridas *in natura* descascadas e frescas, e cozidas em forno. O foco na alimentação e na saúde provocou um intenso interesse em identificar novos alimentos e ingredientes funcionais para prevenir e/ou atenuar doenças específicas (25).

Estudos fitoquímicos têm demonstrado que as folhas e caules do yacon são ricos em proteínas e compostos fenólicos como cafeína, ácidos clorogênicos, ácido ferulico e flavonoides, como a quercetina (26). As raízes da planta contêm frutose, glicose e frutooligossacarídeos, que atuam como prebioticos (27).

Dentro da família dos carboidratos, os fruto-oligossacarídeos (FOS) são conhecidos como oligossacarídeos com unidades repetidas de β - 1,2 – D - Frutofuranosil com um sacarose terminal, e nas raízes do Yacon especificamente, os FOS são compostos por uma cadeia linear de fruto-oligossacarídeos do tipo inulina composto com uma unidade α – (1 → 2) glicose ligada à unidades de frutose β - D - (1 → 2) (28) compondo 34-55% da porcentagem total dos carboidratos presentes na raiz, juntamente com a composição nutricional de 7-9% de glicose, 13-14% de frutose, 10-13% de sacarose, 2.22% de proteínas, minerais como Ca^{2+}, K e Mg^{3+} e outros totalizando 2.40% de teores de cinzas (29) e características físico-químicas como 69,5% de água, 8,0 % de teor de matéria seca, 0,87% de teor de sólidos insolúveis em água e sólidos solúveis em 7,6 ˚Brix (30).

Adicionalmente, compostos bioativos dentro da família dos polifenóis como ácido clorogênico, ácido cafeico, ácido cumárico, ácido protocatequico e aminoácido triptofano são encontrados nas raízes do Yacon (31). Diferentemente das raízes tuberosas, as folhas do Yacon possuem maior porcentagem de umidade (% 83.2), maiores teores de proteínas (% 2.87), cinzas (% 2.68), lipídeos (% 1.24) e menores teores por sua vez, de sacarídeos (% 1.44) e fibras (% 1.68) (32,33). O perfil fitoquímico das folhas do Yacon dos compostos bioativos são na forma de íons polifenóis de ácidos graxos saturados e poli-insaturados como dihexose e derivados de ácidos palmítico, oleico e linoleico (34), conjuntamente com lactonas de sesquiterpeno (35) e óleo essenciais como β-Pineno, β-Cariofileno e γ- Cadineno (36).

Uma série de estudos atuais avaliou o efeito do extrato bruto de folhas e raízes de yacon, obtidos de diferentes maneiras, sobre a glicemia de animais diabéticos (37–40). Honoré et al.,

(2011) (40) relata que a diminuição significante nos níveis de glicemia pós-prandial pode ser explicado pela estimulação de mecanismos pancreáticos, regeneração ou proteção das células β que foram parcialmente destruídas com a utilização de estreptozotocina como indução do DM1 experimental, potencialização da secreção de insulina e provável aumento da utilização periférica de glicose (37,41–43). Baseado nesses trabalhos, atribui-se um efeito antioxidante e anti-diabetogênico do yacon sobre as células β-pancreáticas. Testes realizados por Aybar (2001) (37) com o chá da folha de yacon e com a raiz na forma de suplemento, não evidenciaram qualquer potencial genotóxico nem toxicidade subcrônica. E em estudos recentes, nosso grupo verificou que o tratamento com extrato hidroetanólico das folhas de Yacon promoveu melhora do perfil glicêmico e lipídico, aumento da atividade de enzimas antioxidantes nos músculos esquelético (44) e cardíaco, melhora das alterações relacionadas à cardiomiopatia diabética (organização celular e fibrose) e prevenção/regeneração da arquitetura das ilhotas de Langherans (dados ainda não publicados).

Dessa forma, justifica-se pesquisas sobre a participação de alimentos e plantas medicinais com compostos bioativos que atuem na resposta glicêmica, de forma a atenuar quadro de estresse oxidativo, desde que esses compostos não apresentem efeitos de toxicidade, tornando-se, portanto, terapias com possível potencial antidiabético.

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Capítulo II

Artigos Científicos

CAPÍTULO II

“Recovery of cardiac remodeling and dysmetabolism by pancreatic islet injury improvement in diabetic rats after Yacon leaves extract treatment”.

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Recovery of cardiac remodeling and dysmetabolism by pancreatic islet injury improvement in diabetic rats after Yacon leaves extract treatment

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Abstract

Type 1 diabetes mellitus (T1DM) occurs by destruction of pancreatic β-cells, leads to insulin deficiency and loss of glycemic control, resulting in increased fatty acids (FA) utilization as energy source. FA over-utilization leads to oxidative stress in the cardiomyocytes which is related to diabetic cardiomyopathy. Since the diabetic cardiomyopathy is tightly related to the oxidative stress, the treatment with Yacon leaves could be useful in decrease/prevent the progression of diabetic cardiomyopathy. The aim of this study was to investigate the protective effect of Yacon leaves extract (YLE) on dysmetabolism and cardiomyopathy in diabetic rats. The rats (10/group) were allocated as follows: C, controls; C+Y, controls treated with YLE; DM, diabetic controls; and DM+Y, diabetic rats treated with YLE. T1DM was induced by administration of streptozotocin. treated groups received 100 mg/kg YLE daily via gavage for 30 d. The YLE group improves dysmetabolism in diabetic condition (DM vs. DM+Y), by ameliorating the pancreatic islet injury, insulin release and hyperglycemia, as well as increasing the activity of the antioxidant enzymes and decreasing the fibrosis and cellular disorganization in cardiac tissue. The apparent benefits of YLE seem to be mediated by ameliorating dysmetabolism and oxidative stress in pancreas and cardiac tissue.

Key words: Diabetes mellitus, cardiomyopathy, oxidative stress, hydroethanolic extract, Yacon, pancreas.

Introduction

Type 1 diabetes mellitus (T1DM) is a chronic state of insulin deficiency which results from the destruction of β-cells by the immune system, leading to a structural disorganization of the pancreatic islets. The Epidemiology of Diabetes Interventions and Complications study showed that intensive blood glucose control reduces the risk of several diseases, especially those related to the cardiac tissue [1].

Several studies have demonstrated that development of cardiovascular disease is frequently observed in diabetic patients and in experimental models, and it is one of the major causes that elevate the incidence of morbidity [2–5] . Basically, in diabetic hearts, there is a dramatic shift of the glucose utilization and almost complete reliance on fatty acids oxidation for energy production, resulting in loss of metabolic flexibility as well as morphological changes in the cardiomyocytes [3,6]. In summary, the dysmetabolism in diabetic heart leads to several biochemical and molecular pathways alterations [7]. Deregulated metabolism may be linked to increased production of reactive oxygen species (ROS) that leads to oxidative damage of DNA, proteins and lipids as well as activating stress-sensitive pathways and development of cardiac oxidative stress in diabetes [8,9].

The use of medicinal plants and herbs, for the treatment of many chronic diseases such as diabetes and its complications, has been recognized by a number of scientists and physicians based on their therapeutic properties [6]. Additionally, based on recommendations of the World Health Organization [10], anti-diabetic agents derived from plants are an important alternative, as co-therapy, for the treatment of this condition. Over the past years, great attention has been given in using natural products, based on their pharmacological purposes, as a form of complementary therapy.

Yacon (*Smallanthus sonchifolius*) is a perennial plant originally cultivated in the Andean highlands of South America, and its tubers are commonly used as a food [11]. A number of studies have demonstrated the presence of large amounts of phenolic compounds in extracts from Yacon leaves and tubers [12,13]. So, since the diabetic

cardiomyopathy is tightly related to the oxidative stress – originally from persistent hyperglycemia and the metabolic shift in the cardiac tissue [14,15], the treatment with Yacon leaves can useful in diminishing oxidative damage and decrease or prevent the progression of diabetic cardiomyopathy. Based on these information, the aim of the present study was to investigate the protective effect of Yacon leaves treatment on STZ-induced dysmetabolism and cardiomyopathy based on its antioxidant properties.

Material and Methods

Plant material and extract preparation

S. sonchifolius specimen was cultivated in the Department of Plant Science and Crop Protection, Federal University of Paraná, Curitiba, Paraná, Brazil. Briefly, the leaves from S. sonchifolius were dried for seven days at 50°C, powdered (3 μm), and subjected to percolation at room temperature using a mixture of ethanol : H_2O (7 : 3, v/v) with a flux of 2.0 mL/min/kg. The solvents were evaporated to dryness under a low pressure (45°C) using rotary evaporator in vacuum system to afford the crude Yacon leaves extract (YLE). More information about the plant material, leaves extraction and phytochemical characterization can be found in a previous study of our group [13].

Experimental design

Forty male Wistar rats, 60 d of age, were maintained in an environmentally controlled room (22 ± 3°C; 12-hour light/dark cycle and relative humidity of 60 ± 5%) and were fed with a standard rat pellet diet (Purina Ltd., Campinas, SP, Brazil) and water ad libitum. The experimental protocol was approved by the Ethics Committee on the Use of Animals (CEUA) at the Botucatu Medical School, São Paulo State University (UNESP) under number 1082-2014 (approved in April 24, 2014). The animals were randomly assigned to one of four groups (n= 10): control group (C); control group receiving YLE (C + Y); diabetic rats (DM); and diabetics rats receiving YLE (DM+Y). Diabetes mellitus was induced by i.p. administration of streptozotocin for one time (STZ; 40 mg/body weight). Blood glucose was measured 48 h and 7 days

after the STZ administration. The animals with blood glucose greater than 250 mg/dL were considered diabetic. The animals received YLE (100 mg/kg body weight/day constituted in 1 mL of 0.9% saline) for gavage for 30 days after the 7th day of established diabetic condition. The dose of the treatment was selected based on a previous study conducted by our team [13], where 3 different doses were tested (Y25; Y50; and Y100) and the highest dose presented a better glycemic control (Figure 1). Control animals were given the same volume of saline. The animals were fasted overnight and killed by decapitation after anaesthesia with ketamine (50 mg/kg) and xylazine (0.5 mg/kg) by intraperitoneal injection, and all efforts were made to minimize suffering. Blood was collected in tubes and then centrifuged at 3500 rpm ×g. The serum and heart tissue were collected and stored at −80°C until analysis.

Biochemical and hormonal measurement

An enzymatic colorimetric kit was used to measure serum glucose and triacylglycerol, were measured with an automatic enzymatic analyzer system (Biochemical analyzer BS-200, Mindray, China) with commercial kit (Bioclin®, Belo Horizonte, Minas Gerais, Brazil), non-esterified fatty acids (NEFA) levels was determined by colorimetric kits (WAKO NEFA-C; Wako Pure Chemical Industries, Tokyo, Japan) and Insulin (EMD Millipore Corporation, Billerica, MA, USA) were measured by an immunoassay, using a microplate reader (Spectra Max 190; Molecular Devices, Sunnyvale, CA, USA).

Redox state markers

Preparation of the cardiac tissue for analysis

100 mg of the tissue was homogenized in 1.0 mL of Phosphate buffer saline (PBS) pH 7.4 cold solution ULTRA-TURRAX® T25 basic IKA ® Werke Staufen/Germany, and centrifuged at 800g at 4°C for 10 min. The supernatant was used for the following analysis:

Malondialdehyde (MDA)

Briefly, we added 700 μL of 1% orthophosphoric acid and 200 μL of thiobarbituric acid (42 mM) to the 100 ul supernadant and then boiled it for 60 min in a water bath; the sample was cooled on ice immediately after that. Two hundred μL was transferred to a 2 mL tube containing 200 μL sodium hydroxide-methanol (1 : 12 v/v). The sample was vortex-mixed for 10 s and centrifuged for 3 min at ×g. The supernatant (200 μL) was transferred to a 300 μL glass vial and 50 μL injected onto the column. The HPLC was a Shimadzu LC-10AD system (Kyoto, Japan) equipped with a C18 Luna column (5 μm, 150 × 4.60 mm, Phenomenex Inc., Torrance, CA, USA), a Shimadzu RF-535 fluorescence detector (excitation: 525 nm, emission 551 nm), and 0.5 mL/min flow of phosphate buffer (KH2PO4 1 mM, pH 6.8) [16]. MDA was quantified by area determination of the peaks in the chromatograms relative to a standard curve of known concentrations.

Antioxidants enzymes

Superoxide dismutase activity was measured based on the inhibition of a superoxide radical reaction with pyrogallol and the absorbance values were measured at 420 nm [17]. Catalase activity was evaluated by following the decrease in the levels of hydrogen peroxide. The absorbance values were measured at 240 nm [18]. The activity is expressed as pmole of H2O2 reduced/min/mg protein. Glutathione peroxidase activity was measured by following β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm as described by Flohé and Günzler (1984) [19]; the results were expressed as μmol hydroperoxide reduced/min/mg protein. The values for the enzymes activities were corrected by protein content. Protein was quantified based on Lowry's method[20], using bovine serum albumin as the standard.

Pancreatic histology and pathologic scoring

For histopathological analysis, pancreatic tissue was fixed overnight in 10% formaldehyde, embedded in paraffin, and maintained in 70% ethanol until sliced. After sliced in microtome (4 μm) cross-sections were stained with hematoxylin and

eosin (H&E). Pathology analyses were performed in pancreatic islets by scoring the tissue injury using a method described earlier with some modifications [21]. The total surface of the slide was scored for two different variables determining severity of islet damage, such as size and architecture of islets. The size criterion used defined: Severe, less than 20% of the total field occupation; Moderate, less than 50% of the total field occupation; Slight, less than 70% of the total field occupation; and Normal size islets, occupying around 70-80% of the field. For the architecture criterion: Severe, islets presenting non-symmetric shape and totally disorganized nuclei; Moderate, flat islets and clustered nuclei in the islet periphery; Slight, semi-oval islets with 50% of the nuclei distributed more peripherally; and Normal architecture islets, islets more rounded or oval, nuclei distributed symmetrically throughout the islet.

Immunohistochemistry

The immunohistochemistry procedure was carried following manufacturer protocol, starting with antigen retrieval for paraffin-sectioned slides using standard laboratory protocol. The pancreas slides were incubated in EnVision™ FLEX peroxidase-blocking reagent for 5 minutes to block endogenous enzyme activity. Subsequently, the EnVision™ FLEX anti-insulin primary antibody (1:2000) were incubated for 20 minutes, and after washed to remove primary antibodies, the slides were incubated with EnVision™ FLEX/horseradish peroxidase (HRP) for 20 minutes, followed by two washes, and continuing the procedure incubating the slides with diaminobenzidene (DAB) chromogen for 10 minutes. Finally, the slides were stained with hematoxylin, and dried for xylene preparation. Insulin was quantified through ImageJ® image processing program.

Heart histology

Hearts were harvested for histological evaluation of potential fibrosis, and tissue disorganization. The hearts were fixed in 10% formaldehyde overnight, followed by embedding in paraffin and maintained in 70% ethanol until sliced. The hearts were sliced, with cross sections about 4 μm thick, using a microtome. The staining with H&E and Picrosirius-Red (PSR) were performed according to standard histological

processing. PSR stained sections were used to quantify collagen area using ImageJ® image processing program, following software instructions. Fractal dimension was accessed using H&E stained sections. Three random sections from each animal were photographed through 20X objective, using a light microscope (Leica, German).

Fractal dimension analysis

To quantify the heart nucleus disorganization, H&E stained sections were analyzed using the fractal dimension methodology based on Pacagnelli et al. (2016) description [22]. Using the ImageJ® software, the images of the slides heart tissue were binarized, and the fractal dimension was estimated by using a tool that quantifies pixels distribution in the binarized images; this tool "Fractal box-count" is capable to generate a fractal dimension value (D), which ranges from 0 to 2. A value close to 2 represents more pixel disorganization.

Statistical analysis

For normally distributed data the analyses were performed using two-way ANOVA followed by non-parametric test. For scoring islet damage Linear model for a binomial distribution with logistic link followed by multiple Wald comparison test for islet size and architecture. Statistic tests were performed using SAS for Windows, v. 9.3. Statistic significant were considered when $P<0.05$.

Results

Yacon treatment improves dysmetabolism in diabetic condition

After 30 d of treatment with 100mg/kg/d – based on a dose-response pilot study (Figure 1) –, YLE promoted a significant reduction of 63.39% in the glycemia in the DM+Y group when compared to the untreated group, whereas it increased the insulin concentration in 49.30% in the treated group (Table 1). Additionally, Yacon treatment

decreased the serum TG and NEFA content (DM vs. DM+Y) in 0.39- and 0.43-fold, respectively (Table 1). In the other hand, treatment with Yacon increased the TG content in the control group when compared to the untreated control group.

Yacon treatment ameliorates oxidative stress markers in heart tissue

To investigate the effect of Yacon treatment in oxidative stress response in heart of diabetics, markers of antioxidant defense and oxidative stress were measured for each group. The antioxidant defense enzymes catalase, glutathione peroxidase, and superoxide dismutase levels were significantly decreased in DM compared with the control group, and increased in diabetic animals treated with Yacon when compared with DM (Figure 3a, b, c). In an opposite way, the oxidative stress marker malondialdehyde level was increased in DM group compared to control, and decreased in diabetics treated with Yacon when compared with DM, we also verified an increase in this marker in control group treated with Yacon when compared to untreated control (Figure 3d).

Yacon treatment reduces pancreas severe phenotype and increases insulin production

To analyze the effect of the treatment on the Langerhans islet size and architecture, we used a linear model for binomial distribution with logistic link followed by multiple comparison Wald test. For the analysis of the islets number with normal size, the results showed that the DM group presented fewer islets with normal size compared with the control group, and the treatment with Yacon (DM+Y) increased the number of islets with normal size (Figure 2b). For the slight, moderate and severe alterations, the DM group showed an increase in islets with these characteristics, whereas the treatment seems to prevent these alterations in diabetic group (Figure 2b). Moreover, the architecture analysis showed a reduction in the number of islets with normal architecture. Notably, we verified a higher number of islets with altered architecture (moderate and severe) in DM when compared to DM+Y, suggesting that the treatment with Yacon could alleviates the islets architecture deterioration in diabetic condition. No moderate or severe changes were observed in the control group (Figure 2b). The results description for the islets staging criteria are exposed in Table S1 and

Figure S1 (Supplementary material).

Yacon decreases fibrosis and nuclear disorganization in heart of diabetic rats

To verify the effect of the treatment in diabetic heart fibrosis, we analyzed histological sections stained with PSR, which is responsible for collagen staining. Animals belong to untreated diabetic group present increased collagen area in extracellular matrix compared to control. Furthermore, the diabetic rats treated with Yacon showed a decrease in fibrosis accumulation compared to DM (Figure 4a and c). Moreover, we analyzed heart tissue organization, by assessing fractal dimension of nucleus localization using histological sections stained with H&E. Fractal analysis showed that diabetics have higher nucleus disorganization than control. Additionally, the treatment with Yacon was able to reverse the nuclear organization in diabetics (Figure 4b, d). These data indicate the beneficial effect of Yacon in reduce heart extracellular matrix fibrosis in diabetic condition, and the potential to ameliorate heart tissue disorganization.

Discussion

It is known that the hyperglycemia that occurs in diabetes is the major cause of diabetic complications. Since STZ model of diabetes induction causes destruction of pancreatic β-cells, simulating the physio-pathologic process of the disease, it generates a deficiency in insulin biosynthesis and secretion and, consequently, increase in serum glycemia content since it becomes unavailable to insulin-sensitive tissue. In the present study, diabetic animals underwent to hyperglycemic condition after 7 d of STZ administration (Table 1). These conditions can be better visualized based on the histological analyzes where DM group presented a loss of the architecture and size of the islets (Figure 2a, b) and decrease of insulin production (Figure 2c, d). Additionally, under these conditions, the breakdown of structural protein and lipolysis are increased, promoting weight loss and increase of circulating lipids (e.g. triacylglycerol and fatty acid) [3]. Concomitantly, the serum levels of NEFA were enhanced in the DM group (Table 1), this represents an increase in the availability of NEFA to the heart for energy generation since the cardiac tissue (insulin-dependent) do not utilize glucose adequately

as energy source [23]. An increase in myocardial fatty acid uptake and oxidation has been described in humans with both type 1 and type 2 diabetes, as well as in many animal models [24]. Elevated circulating glucose [25] and free fatty acid [26,27] have been shown to possesses an important role in the complications of both type 1 and type 2 diabetes. In the other hand, the treated diabetic group presented a decrease in serum glucose and a decrease of circulating lipids. This can be attributed to an improvement of the glycemic control, suggesting greater peripheral utilization of glucose and maintenance of the adipose and muscle tissues. These biochemical and hormonal improvements are potentially linked to the preservation and/or regeneration of the remaining pancreatic islet that were partially destroyed by STZ and, consequently, the potentiation of the insulin secretion from the protected/regenerated β-cells [28][29]. In agreement to our data, some authors demonstrated that the hydroethanolic extract of yacon leaves significantly reduced glucose levels in diabetic rats and in genetically type-2 diabetic mice [11,30,31], in parallel, Honoré et al (2012) [32] and Habib et al (2011) [33], respectively demonstrated that immunofluorescent staining of the pancreatic tissues in diabetic animal presented a decrease of insulin density whereas diabetic rats treated with Yacon leaves decoction or Yacon root flour showed strong insulin immunostaining.

In summary, there are extensive evidences that the hyperglycemia established in diabetic condition is associated with the generation of reactive oxygen species (ROS and weakens of the antioxidant defense, resulting in enhanced oxidative stress [13,34]. Indeed, the activity of several antioxidant enzymes is decreased in the diabetic heart in both rats and humans [13,35,36]. Here, we have demonstrated that the activity of the antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase is decreased in diabetic group and MDA concentration are elevated in cardiac tissue (Figure 3a, b, c, d), indicating an oxidative stress process. These enzymes are regarded as the first line of the antioxidant defense system and work together to eliminate ROS generated during oxidative stress [37]. The deleterious effects of oxidative stress in the diabetic heart are well established, including cell death and cardiac fibrosis [38–40]. Cardiac fibrosis is a major feature of diabetic cardiomyopathy [41]. Diabetic cardiomyopathy is defined as a ventricular dysfunction that occurs in diabetic patients not related to another cause (e.g. hypertension or coronary artery disease) [42,4]. Somaratne and colleagues [43] reported that 56% of diabetic patients had diabetic

cardiomyopathy. Although the etiology of the diabetic cardiomyopathy is not yet completely understood, the pathophysiology of this condition is believed to be multifactorial. Existing evidences suggests that persistent hyperglycemia-induced oxidative stress is an important contributor to it [14,15]. In this condition, an excessive production of extra cellular matrix protein leads to increased myocardial hardness and consequent cardiac dysfunction and, consequently, resulting in cardiac failure [44]. Concomitantly, our data indicates that the diabetic condition leads to an increase of collagen deposit and nuclear disorganization in cardiac tissue (Figure 4 a, b, c, d). Diabetic patients have a 2- to 5-fold increased risk of developing heart failure, one of the greatest contributors to morbidity and mortality [45]. Here we've also showed an increase of oxidative stress in the heart of untreated DM group, whereas the Yacon treatment promoted a decrease of oxidative stress marker and an increased of the antioxidant enzymes activity. We have previously demonstrated the antioxidant activity of Yacon leaves in soleus muscle, potentially due the presence of several antioxidant compounds in the extract [13]. Additionally, phytochemical studies of yacon leaves showed the presence of high polarity antioxidant compounds such as caffeic, chlorogenic and three dicaffeoilquinic acids [46]. It is known that compounds such as phenolic acids, polyphenols, and flavonoids scavenge free radicals such as peroxide, hydroperoxide, or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to diabetes complications. So, since oxidative stress is related to the cardiac remodeling and fibrosis through several mechanisms [38,39,47], this could be the mechanism by which DM+Y presented an improvement of cardiac alterations mentioned above. Given the fundamental role of oxidative stress in the pathogenesis of diabetes and diabetic cardiomyopathy, there is growing interest in the use of antioxidants as a complementary therapeutic approach to prevent/treat these conditions. Numerous studies demonstrated that ameliorating oxidative stress through antioxidant treatment might be an effective strategy for reducing diabetic cardiomyopathy [48,49].

The important findings of this study are the cardio and pancreatic protective effects of Yacon treatment in experimental STZ-induced diabetic cardiomyopathy and pancreatic islet dysfunction in terms of preservation of Largerhans islet architecture and insulin production as well as an inhibition of collagen content accumulation and enhancement of antioxidant enzymes activities in cardiac tissue. Summarizing, our results demonstrated that STZ administration successfully induced diabetes and diabetic

cardiomyopathy as indicated by the fractal analysis, indicating cellular disorganization, as well as increase in collagen deposition in heart tissue and decrease of insulin production and preservation of the architecture of the pancreatic β-cells. Interestingly, these cardiac and pancreatic abnormalities were improved by the administration of Yacon extract. Furthermore, exploring potential therapeutic effects of plants and herbal medicine could contribute to detect new targets and treatments. Our findings, even if in basic research model, provide information that can guide future studies aimed at elucidating new therapeutic alternatives for diabetic complications.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Supplementary Material

Table S1. Analysis of the islets by scores. Description of the results for the islets staging criteria (normal, slight, moderate and severe) obtained after the linear model for a binomial distribution with logistic link followed by multiple Wald comparison test for islet size and architecture.

		C vs. DM	C vs. C+Y	C+Y vs. DM+Y	DM vs. DM+Y
Size	**Normal**	P < 0.001*	ND or NT	P < 0.001*	P < 0.001*
	Slight	P = 0.0094*	ND or NT	ND or NT	P < 0.001*
	Moderate	P < 0.001*	ND or NT	ND or NT	P = 0.025*
	Severe	ND or NT	ND or NT	ND or NT	P = 0.0017*
Architecture	**Normal**	P < 0.001*	ND or NT	P < 0.001*	P < 0.001*
	Slight	ND or NT	ND or NT	ND or NT	P = 0.004*
	Moderate	ND or NT	ND or NT	ND or NT	P < 0.001*
	Severe	ND or NT	ND or NT	ND or NT	ND or NT

C: Control; C+Y: Control treated with Yacon; DM: Diabetes Mellitus; DM+Y: Diabetes Mellitus treated with Yacon; ND: No difference; NT: Not tested. The linear model for a binomial distribution followed by multiple Wald comparison test analyzed statistical significance.

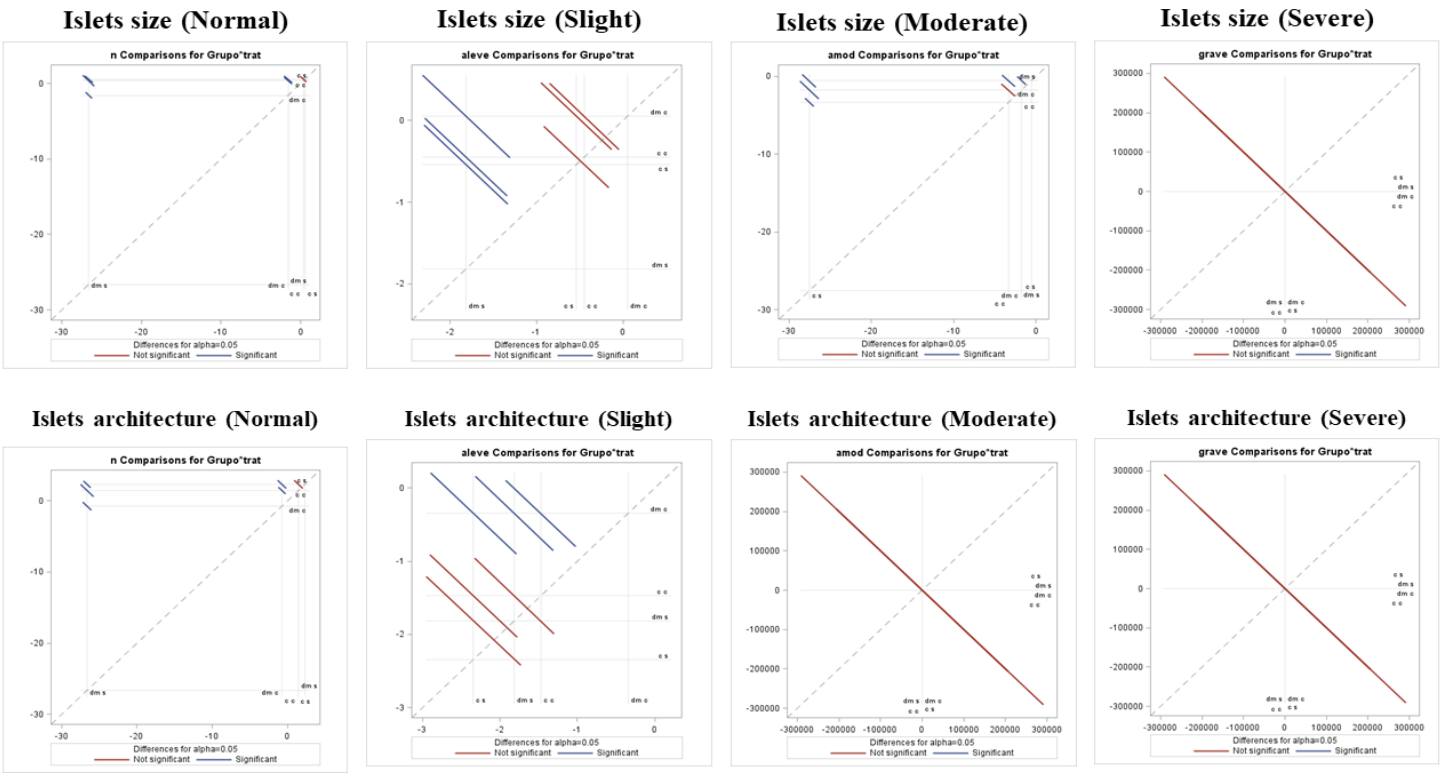

Figure S1. Analysis of the islets by scores. Description of the results for the islets staging criteria (normal, slight, moderate and severe) obtained after the linear model for a binomial distribution with logistic link followed by multiple Wald comparison test for islet size and architecture. Blue lines represent statistical significance while red lines mean no difference (ND) or not tested (NT) among the groups.

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Figures, tables and figure legends.

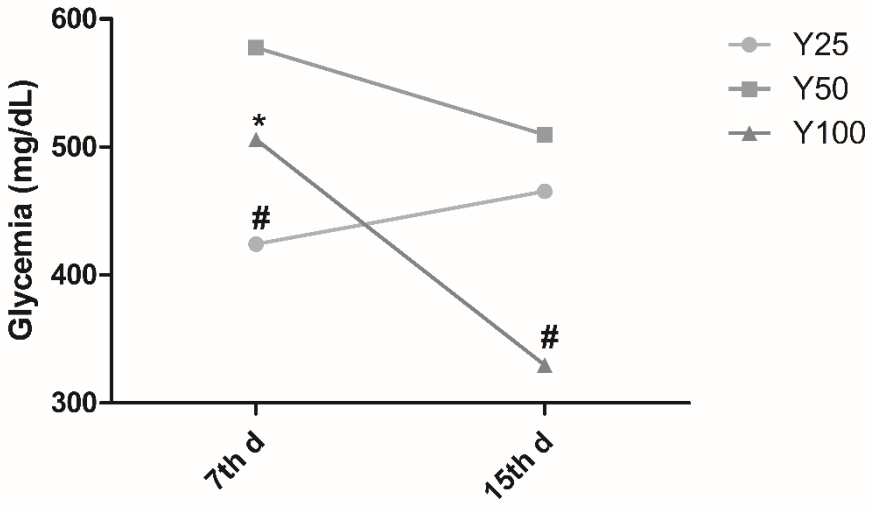

Figure 1. Dose-response profile of Yacon leaves. The animals were randomly assigned to one of three groups: Y25; Y50; and Y100 (25, 50, and 100 mg/kg body weight/day of Yacon extract constituted in 1 mL of 0.9% saline, respectively). Diabetes mellitus was induced by one i.p. administration of streptozotocin (STZ; 40 mg/body weight), and the animals received HEYL for gavage for 15 days after the establishment of diabetic condition and glycemia was measured at day 7 (7^{th} d) and day 15 (15^{th} d). C: Control; C+Y: Control treated with Yacon; DM: Diabetes Mellitus; DM+Y: Diabetes Mellitus treated with Yacon. The data are represented as the median. Statistical analysis was performed using generalized linear model, and one-way analysis of variance test. Significant values are represented by $P<0.05$. * vs. Y25; # vs. Y50.

Table 1. Serum biochemical and hormonal outcomes.

	Groups			
Outcomes	**C**	**C+Y**	**DM**	**DM+Y**
Initial Glycemia ***mg/dL***	128 ± 14.75	119.6 ± 14.76	416.11 ± 15.55 *	395.33 ± 19.05 #
Final Glycemia ***mg/dL***	94 ± 17.90	88.66 ± 18.87	299.75 ± 20.02 *	109.71 ± 19.46 †
Insulin ***pmol/L***	32.25 ± 2.45	30.87 ± 2.60	20.24 ± 3.30 *	30.22 ± 3.01 †
TG ***mg/dL***	54.74 ± 3.26	71.53 ± 3.64 *	89.77 ± 3.89 *	64,42 ± 4.21 †
NEFA ***mEq/L***	0.34 ± 0.03	0.38 ± 0.03	0.43 ± 0.03	0.30 ± 0.03 †

The data are represented as the mean ± SEM. Statistical analysis was performed using generalized linear model, and two-way analysis of variance test complemented with nonparametric test. Significant values are represented by P<0.05. * vs. C; # vs. C+Y; † vs. DM. C: Control; C+Y: Control treated with Yacon; DM: Diabetes Mellitus; DM+Y: Diabetes Mellitus treated with Yacon; TG: triacylglycerol; NEFA: non-esterified fatty acid.

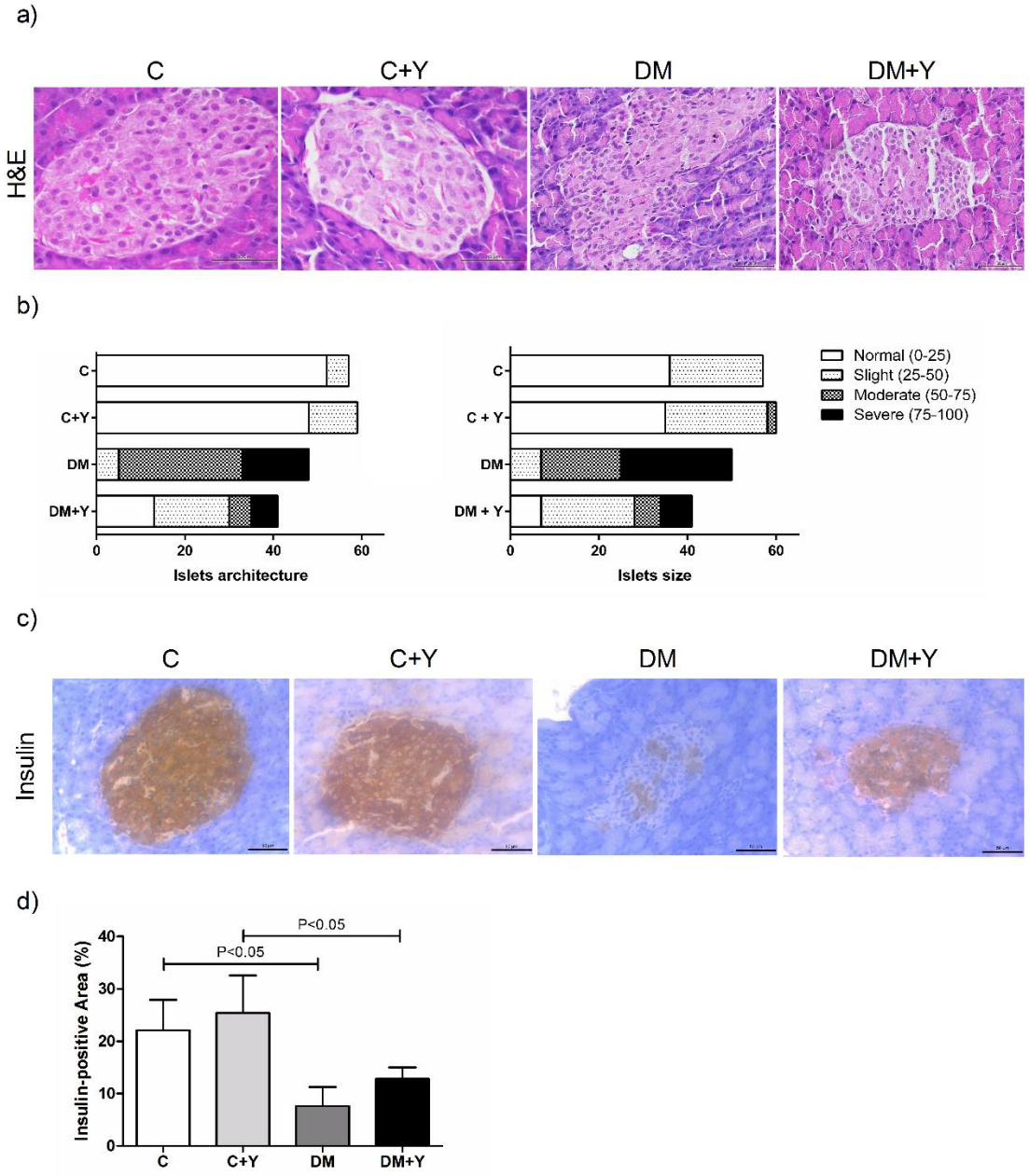

Figure 2. Pancreatic histology, pathologic scoring and immunohistochemistry analysis. (a) Pancreas histological sections stained with Hematoxylin and Eosin. (b) Pancreas scoring among groups using as parameter islets architecture, and islets size. (c) Histological analysis of the pancreas by immunohistochemistry for insulin. (d) Quantitative analysis of the positive area for insulin in the pancreatic islets quantified using the imaging software ImageJ. Original magnification x20. Scale bars, 50 μm. The data represent the mean ± standard deviation. Statistical analysis was performed using generalized linear model, and two-way analysis of variance test complemented with nonparametric test for insulin-positive area or generalized

linear model (binomial) followed by Wald test for scoring analysis. Significant values were represented by P<0.05. C: Control; C+Y: Control treated with Yacon; DM: Diabetes Mellitus; DM+Y: Diabetes Mellitus treated with Yacon; H&E: Hematoxylin and Eosin.

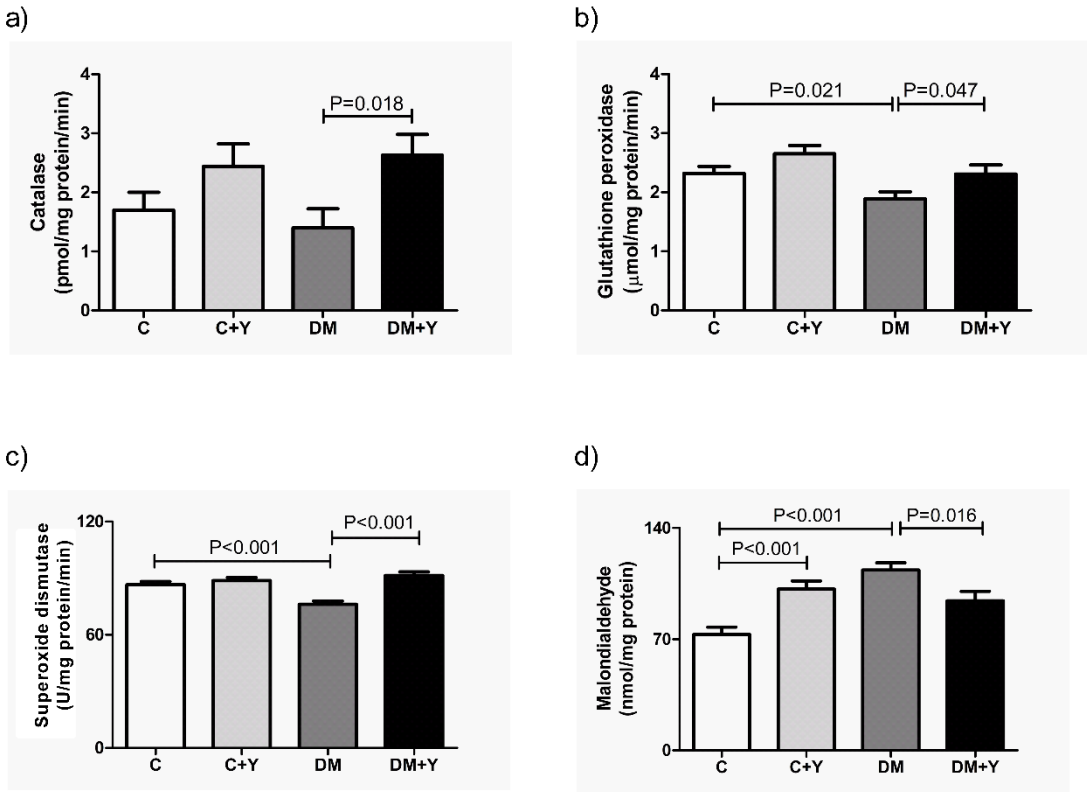

Figure 3. Redox state markers. (a) Catalase; (b) Glutathione peroxidase; (c) Superoxide dismutase activities; and (d) Malondialdehyde concentration in the cardiac tissue. The data are represented as the mean ± SEM. Statistical analysis was performed using generalized linear model, and two-way analysis of variance test complemented with nonparametric test. Significant values are represented by P<0.05. * vs. C; # vs. C+Y; † vs. DM. C: Control; C+Y: Control treated with Yacon; DM: Diabetes Mellitus; DM+Y: Diabetes Mellitus treated with Yacon.

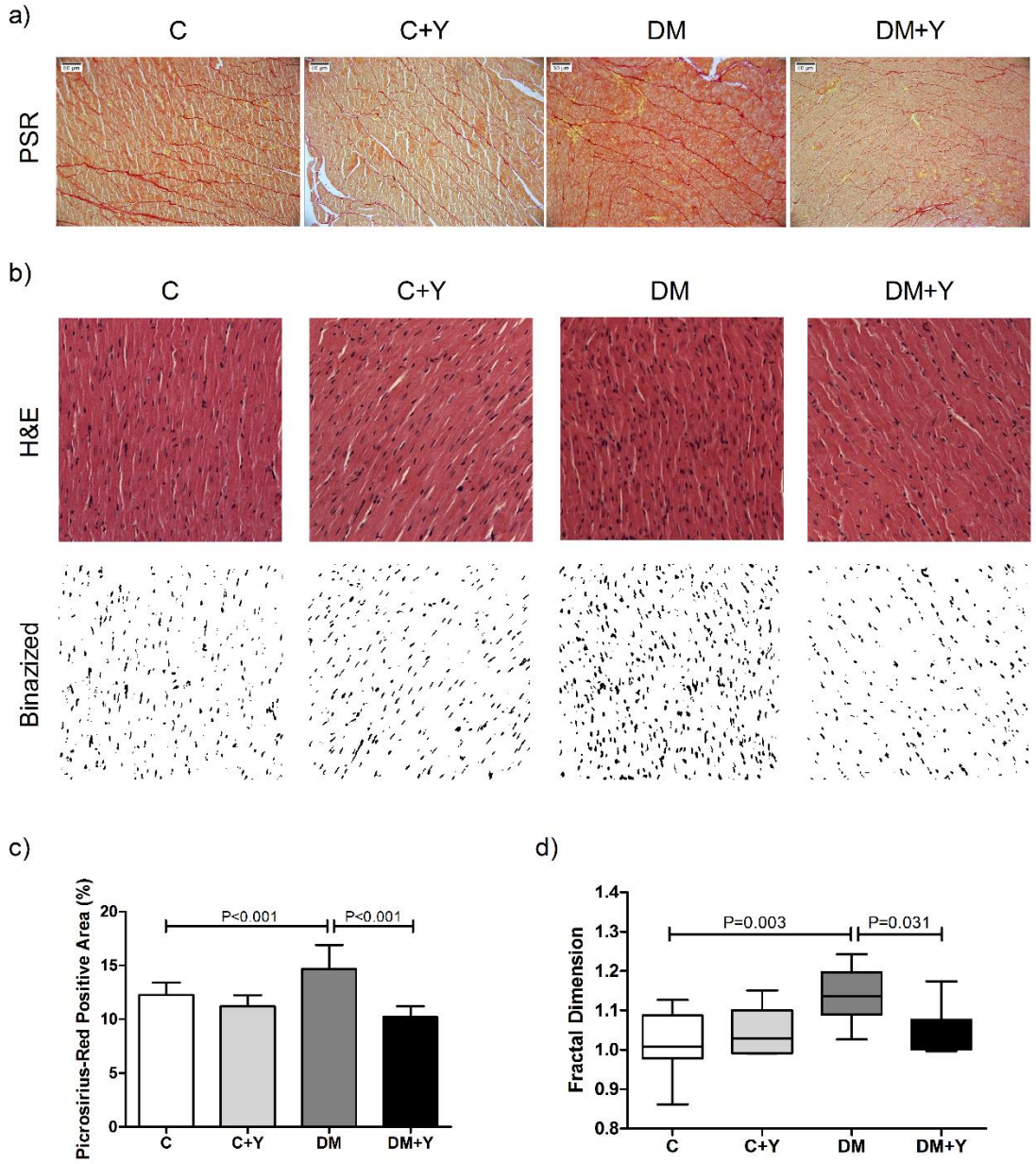

Figure 4. Extracellular matrix collagen area and nuclear disorganization in the cardiac tissue. (a) Heart histological sections of control, diabetic and treated animals stained with Picrosirius-red to access extracellular matrix fibrosis. (b) Histological sections of the heart stained with Hematoxylin and Eosin and corresponding image after binarization of each group. Original magnification x20. Scale bars, 50 μm. (c) Quantitative analysis of collagen area in the PSR-stained sections. (d) Fractal dimension quantified by imaging software ImageJ. The data represent the mean ± standard deviation, and for fractal analysis data are expressed as box plot graphic showing first and third quartile, median, minimum and maximum. Statistical analysis was performed using two-way analysis of variance test complemented with nonparametric test. Significant values were represented by $P<0.05$. C: Control; C+Y: Control treated with Yacon; DM: Diabetes Mellitus; DM+Y: Diabetes Mellitus treated with Yacon; PSR: Picrosirius-red; H&E: Hematoxylin and Eosin.

CAPÍTULO II

“Yacon (*Smallanthus sonchifolius*) Leaf Extract Attenuates Hyperglycemia and Skeletal Muscle Oxidative Stress and Inflammation in Diabetic Rats”.

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Research Article

Yacon (*Smallanthus sonchifolius*) Leaf Extract Attenuates Hyperglycemia and Skeletal Muscle Oxidative Stress and Inflammation in Diabetic Rats

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The effects of hydroethanolic extract of Yacon leaves (HEYL) on antioxidant, glycemic, and inflammatory biomarkers were tested in diabetic rats. Outcome parameters included glucose, insulin, interleukin-6 (IL-6), and hydrophilic antioxidant capacity (HAC) in serum and IL-6, HAC, malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in soleus. The rats (10/group) were divided as follows: C, controls; C + Y, HEYL treated; DM, diabetic controls; and DM + Y, diabetic rats treated with HEYL. Diabetes mellitus was induced by administration of streptozotocin. C + Y and DM + Y groups received 100 mg/kg HEYL daily via gavage for 30 d. Hyperglycemia was improved in the DM + Y versus DM group. Insulin was reduced in DM versus C group. DM rats had higher IL-6 and MDA and lower HAC in the soleus muscle. HEYL treatment decreased IL-6 and MDA and increased HAC in DM rats. DM + Y rats had the highest CAT activity versus the other groups; GPx was higher in C + Y and DM + Y versus their respective controls. The apparent benefit of HEYL may be mediated via improving glucoregulation and ameliorating oxidative stress and inflammation, particularly in diabetic rats.

1. Introduction

Type 1 diabetes mellitus (T1DM) occurs by autoimmune-mediated destruction of pancreatic β-cells, leading to insulin deficiency and loss of glycemic control. The hyperglycemia that occurs in diabetes increases the production of reactive oxygen species (ROS) and weakens antioxidant defense, resulting in enhanced oxidative stress [1]. ROS mediate several biochemical and molecular pathways that can exacerbate oxidative stress [2], such as activating the transcription factor nuclear factor kappa B (NF-κB), which increases the transcription of inflammatory cytokines and chemokines [3] promoting inflammation. Moreover, uncontrolled ROS generation could also attack the cellular proteins, lipids, and nucleic acids leading to cellular dysfunction including loss of energy metabolism, alteration on cell signaling and cell cycle control, mutations, and inflammation. In addition, it plays a role in several pathological processes in skeletal muscle [4]. These reactive species are important signaling molecules necessary for muscle function and for adaptive response to stress [5]. However, overproduction of ROS and decrease of the antioxidant defense have negative impact on muscle function, as impaired muscle growth and strength and altered metabolic capacity [6].

Medicinal plants are widely used as alternative therapeutics for the prevention or treatment of diseases. Recently, great attention has been paid to the use of natural compounds, due to their nutritional and pharmacological characteristics [7].

Yacon (*Smallanthus sonchifolius* [Poepp. & Endl.] H. Robinson, Asteraceae) is a native Andean plant cultivated for its tubers, which are commonly used as a food in South America. Some studies have reported the presence of large amounts of phenolic compounds in extracts from Yacon leaves and tubers, mainly chlorogenic, protocatechuic, ferulic, rosmarinic, gallic, gentisic, and caffeic acids and their derivatives [8]. Evidence has also emerged about the antioxidant activity [9], protective effects on oxidative damage and glucose metabolism in rat hepatocytes, and insulin-like effects of Yacon leaf extracts [10, 11].

Antioxidant compounds have long been known to diminish inflammatory and oxidative stress responses. In addition, antioxidants scavenge ROS and increase the capacity of the antioxidant defense enzyme system [2]. Therefore, antioxidants can help diminish oxidative damage and inflammation and slow or prevent the progression of diabetic complications.

Natural products are the groundwork of preventing and curing several diseases. Moreover, ethnopharmacological knowledge is one attractive way to enhance the probability of success in new drug-finding efforts. Regarding diabetic complications, plants that can be effectively used based on their therapeutic applications, for example, for diabetes, are worthy of special attention, and studies are needed regarding their side effects, the ability to maintain normal levels of glycemia, and their possible control on oxidative stress and inflammation. Considering the complications of type 1 diabetes and previous data on Yacon's activities, the present study was undertaken to elucidate the antioxidant, anti-inflammatory, and antihyperglycemic activity of hydroethanolic extract from *S. sonchifolius* leaves (HEYL) in the serum and skeletal muscle of STZ-induced diabetic rats.

2. Materials and Methods

2.1. Plant Material and Extract Preparation. The leaves of *S. sonchifolius* were collected in June (2014) by Klinsmann Carolo dos Santos in Curitiba, PR, Brazil. The specimen was provided by Dr. Átila Francisco Mógor from the Department of Plant Science and Crop Protection, Federal University of Paraná, Curitiba, Paraná, Brazil, and identified by Dr. Lin Chau Ming from São Paulo State University (UNESP), School of Agriculture, Botucatu, SP, Brazil, and the voucher specimen was deposited to the Herbarium at the São Paulo State University (UNESP), Institute of Biosciences, Botucatu, SP, Brazil, under the register 32752, for future reference. The leaves from *S. sonchifolius* were dried for seven days at 50°C, powdered (3 μm), and subjected to percolation at room temperature using a mixture of ethanol : H_2O (7 : 3, v/v) with a flux of 2.0 mL/min/kg. The solvents were evaporated to dryness under a low pressure (45°C) using rotary evaporator in vacuum system to afford the crude HEYL.

2.2. Characterization of Phenolics. For the characterization of phenolics, 10 mg HEYL was reconstituted in 1 mL methanol, followed by acidic hydrolysis with 1 mL of 2.4 M HCl at 80°C for 2 h in the dark. After the incubation, the solution was filtered through a 0.45 μm nylon membrane (Millipore Corp., Bedford, MA) and then injected onto a HPLC system equipped with a Zorbax SB-C18 column (4.6 × 250 mm, 3.5 μm) and a CoulArray 5600A electrochemical detector (ESA Inc., Chelmsford, MA). Phenolic acids and flavonoids were quantified according to the method of Li et al. (2009) [12]. The limits of quantitation for phenolic acid and flavonoids were 1 ng on column. The linearity of calibration curves of authentic standards with concentrations ranging from 0.01 to 2 ng/ml was at least ≥0.991. The identification of each compound was based on a comparison of the retention time and electrochemical response of the authenticated standards. The results are expressed in μg/100 mg HEYL.

2.3. Dose-Response Profile of HEYL Treatment. To establish a dose-response profile for the antihyperglycemic activity of Yacon leaves, we used varying doses of HEYL (25, 50, and 100 mg/kg body weight/day constituted in 1 mL of 0.9% saline) to identify the lowest dose that could elicit an optimal antihyperglycemic effect. Fifteen male Wistar rats, 60 d of age, were maintained in an environmentally controlled room (22 ± 3°C; 12-hour light/dark cycle and relative humidity of 60 ± 5%) and were fed with a standard rat pellet diet (Purina Ltd., Campinas, SP, Brazil) and water ad libitum. The animals were randomly assigned to one of three groups: HEYL 25; HEYL 50; and HEYL 100. *Diabetes mellitus* was induced by one i.p. administration of streptozotocin (STZ; 40 mg/body weight), and the animals received HEYL for gavage for 2 weeks after the establishment of diabetic condition. We found that after 2 weeks of the treatment, the highest dose showed the most potent hyperglycemic effect in STZ model of diabetes. Thus, the subsequent experiments with HEYL were carried out with the dose of 100 mg/kg administered orally.

2.4. Animals and Experimental Groups. Forty male Wistar rats, 60 d of age, were maintained in an environmentally controlled room (22 ± 3°C; 12-hour light/dark cycle and relative humidity of 60 ± 5%) and were fed with a standard rat pellet diet (Purina Ltd., Campinas, SP, Brazil) and water ad libitum. The experimental protocol was approved by the Ethics Committee on the Use of Animals (CEUA) at the Botucatu Medical School, São Paulo State University (UNESP) under number 1082-2014 (approved in April 24, 2014). The animals were randomly assigned to one of four groups ($n = 10$): C (control group): normal rats; C + Y: normal rats receiving HEYL; DM: diabetic rats; and DM+Y: diabetics rats receiving HEYL. *Diabetes mellitus* was induced by i.p. administration of streptozotocin for one time (STZ; 40 mg/body weight). Blood glucose was measured 48 h and 7 days after the STZ administration. The animals with blood glucose greater than 250 mg/dL were considered diabetic. The animals received HEYL (100 mg/kg body weight/day constituted in 1 mL of 0.9% saline) for gavage for 30 days after the 7th day of established diabetic condition. Control animals were given the same volume of saline. The animals were fasted overnight and killed by decapitation

after anaesthesia with ketamine (50 mg/kg) and xylazine (0.5 mg/kg) by intraperitoneal injection, and all efforts were made to minimize suffering. Blood was collected in tubes and then centrifuged at 3500 rpm ×g. The serum and soleus muscle were collected and stored at −80°C until analysis.

2.5. Preparation of the Soleus Muscle for Analysis. Soleus muscle was weighed (100 mg) and homogenized in 1.0 mL cold PBS (pH 7.4) using ULTRA-TURRAX® T25 basic IKA® Werke Staufen/Germany. After centrifugation at 800 ×g at 4°C for 10 min, the supernatant was collected for malondialdehyde (MDA) and IL-6 determinations. For the antioxidant enzymes determination, 100 mg soleus muscle was homogenized (1 : 10 v/v) in KH_2PO_4 (10 mmol/L)/KCl (120 mmol/L), pH 7.4, and centrifuged at 2.000 ×g for 20 min.

2.6. Biochemical Measurements in Serum and Soleus Muscle. An enzymatic colorimetric kit was used to measure serum glucose (Bioclin®, Belo Horizonte, Minas Gerais, Brazil). Insulin (Immuno-Biological Laboratories, Inc.) and IL-6 (R&D Systems, Inc.) were measured by an immunoassay, using a microplate reader (Spectra Max 190; Molecular Devices).

2.7. Pancreatic Beta-Cell Function. Pancreatic beta-cell function was determined using the index of homeostasis model assessment (HOMA) [13] using the following formula: HOMA-BETA (*Homeostasis Model Assessment* Beta-*Cell Function*) = 20 × Fasting Insulin (μU/mL)/Fasting Glucose (mM) − 3,5.

2.8. Malondialdehyde (MDA) Analysis in Soleus Muscle. A 100 μL aliquot of soleus muscle homogenate was used for MDA analysis. Briefly, we added 700 μL of 1% orthophosphoric acid and 200 μL of thiobarbituric acid (42 mM) to the sample and then boiled it for 60 min in a water bath; the sample was cooled on ice immediately after that. Two hundred μL was transferred to a 2 mL tube containing 200 μL sodium hydroxide-methanol (1 : 12 v/v). The sample was vortex-mixed for 10 s and centrifuged for 3 min at 13,000 ×g. The supernatant (200 μL) was transferred to a 300 μL glass vial and 50 μL injected onto the column. The HPLC was a Shimadzu LC-10AD system (Kyoto, Japan) equipped with a C18 Luna column (5 μm, 150 × 4.60 mm, Phenomenex Inc., Torrance, CA, USA), a Shimadzu RF-535 fluorescence detector (excitation: 525 nm, emission 551 nm), and 0.5 mL/min flow of phosphate buffer (KH_2PO_4 1 mM, pH 6.8) [14]. MDA was quantified by area determination of the peaks in the chromatograms relative to a standard curve of known concentrations.

2.9. Measurement the Hydrophilic Antioxidant Capacity (HAC) in Serum and Soleus Muscle. The hydrophilic antioxidant capacity was determined fluorometrically, as described by Beretta et al. (2006) [15] using a VICTOR X2 reader (Perkin Elmer, Boston, MA). The antioxidant activity was quantitated by comparing the area under the curve relating to the oxidation kinetics of the suspension phosphatidylcholine (PC), which was used as reference biological matrix. The peroxyl radical 2′,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) was used as an initiator of the reaction. The results represent the percent inhibition (4,4 difluoro-5-(4-phenyl 1-3 butadiene)-4-bora-3,4-diaza-s-indacene) (BODIPY) 581/591 plasma with respect to the control sample of BODIPY 581/591 PC liposome. All analyses were performed in triplicate. The results are reported as percentage of protection.

2.10. Antioxidant Enzymes Activity Evaluation in Soleus Muscle. Superoxide dismutase activity was measured based on the inhibition of a superoxide radical reaction with pyrogallol and the absorbance values were measured at 420 nm [16]. The values are expressed as units per milligram of protein. Catalase activity was evaluated by following the decrease in the levels of hydrogen peroxide. The absorbance values were measured at 240 nm [17]. The activity is expressed as pmole of H_2O_2 reduced/min/mg protein. Glutathione peroxidase activity was measured by following β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm as described by Flohé and Günzler (1984) [18]; the results were expressed as μmol hydroperoxide reduced/min/mg protein. Protein was quantified based on Lowry's method [19], using bovine serum albumin as the standard.

2.11. Statistical Analysis. Results are expressed as mean and standard error of the mean (SEM), and significance was calculated by two-way ANOVA followed by Holm-Sidak method. The software used was SigmaStat version 3.5 for Windows (Systat Software, Inc., San Jose, CA, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Glycemia, Insulin, and HOMA-BETA. STZ-induced diabetic rats (DM and DM + Y) showed 3.25- and 3.08-fold, respectively, higher blood glucose levels than the control groups (Figure 1(a)) in the beginning of the experiment (7 d after administration of STZ). After treatment with HEYL, the DM + Y animals showed reduction of glycemia to values similar to the controls (Figure 1(b)). The insulin was lower in DM when compared with the control groups (Figure 1(c)). The same was found for HOMA-BETA; DM presented the lowest values when compared to C and DM + Y, whereas the treated DM group presented increase of HOMA-BETA (Figure 1(d)).

3.2. Characterization of Phenolics. Ten phenolics in the hydroethanolic extract of Yacon were quantified using a HPLC-ECD method. The active principles with their concentrations, retention time (RT), and peak area (μC) are presented in Table 1.

3.3. Antioxidant Enzymes and Lipoperoxidation Marker in Soleus Muscle. The treatment with Yacon leaves in DM + Y decreased MDA (Figure 3(d)) in soleus muscle when compared to DM, which presented the highest values for this variable. DM + Y group presented the highest catalase

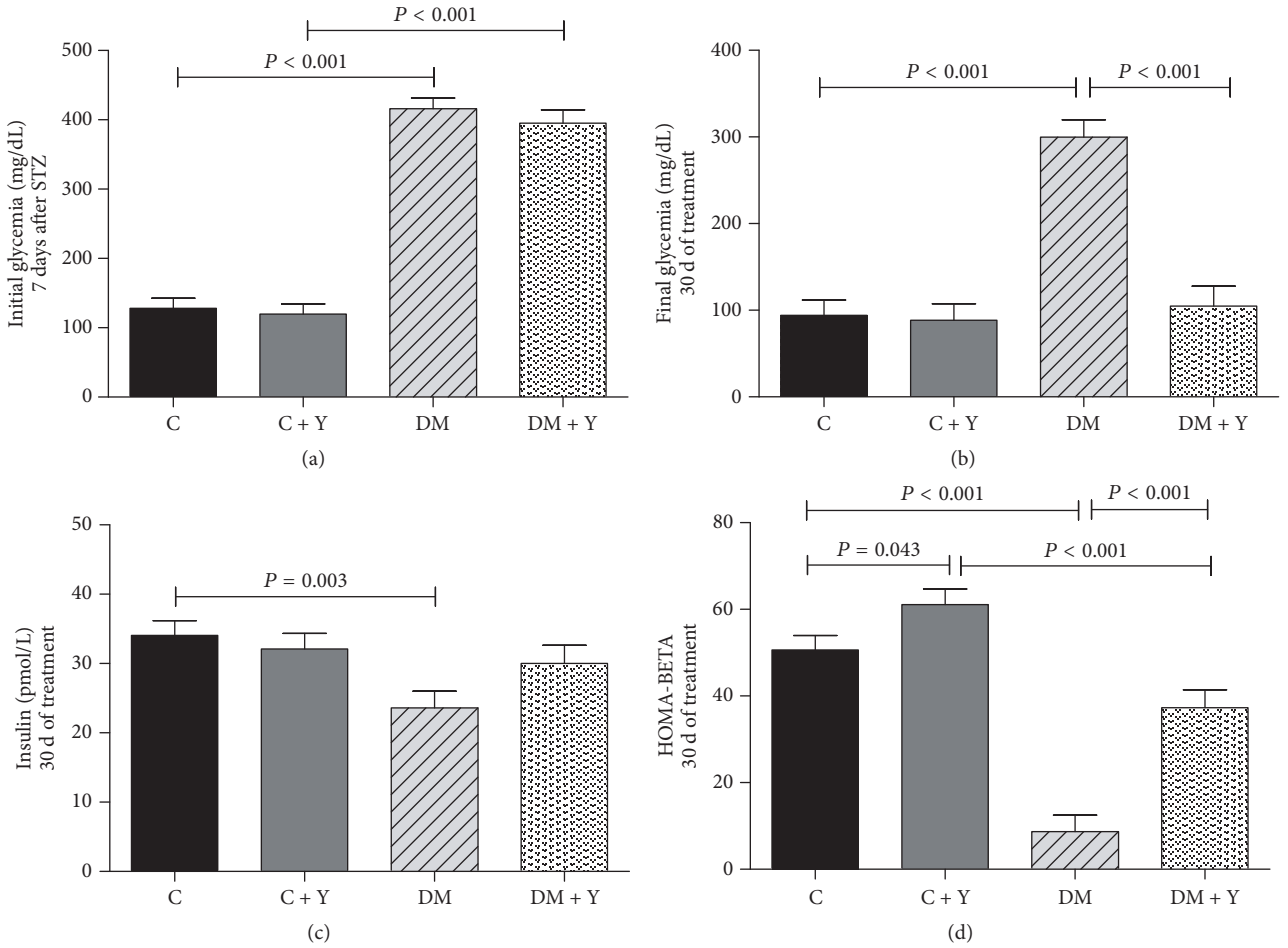

Figure 1: (a) Initial glycemia (7 d after STZ administration); (b) final glycemia (30 d of treatment); (c) insulin (30 d of treatment); (d) HOMA-BETA (30 d of treatment) of the different experimental groups. C (control group): normal rats; C + Y: normal rats receiving HEYL; DM: diabetic rats; and DM + Y: diabetics rats receiving HEYL. The results are expressed as the mean ± SEM.

Table 1: Phenolic content of hydroethanolic extract of Yacon leaves.

RT (min)	Name	Peak area (μC)	Concentration
15.29	Protocatechuic acid	6.98	10.11
25.83	Gentisic acid	8.78	7.64
33.13	Chlorogenic acid	2.30	8.17
35.97	Vanillic acid	3.08	1.34
37.65	Caffeic acid	66.10	27.56
42.25	Epicatechin	2.20	5.11
46.67	p-Coumaric acid	19.50	169.81
49.78	Ferulic acid	7.05	13.21
50.43	Sinapic acid	6.41	4.68
75.35	Quercetin	128.0	399.00

RT: retention time. Concentration is expressed in μg/100 mg of HEYL.

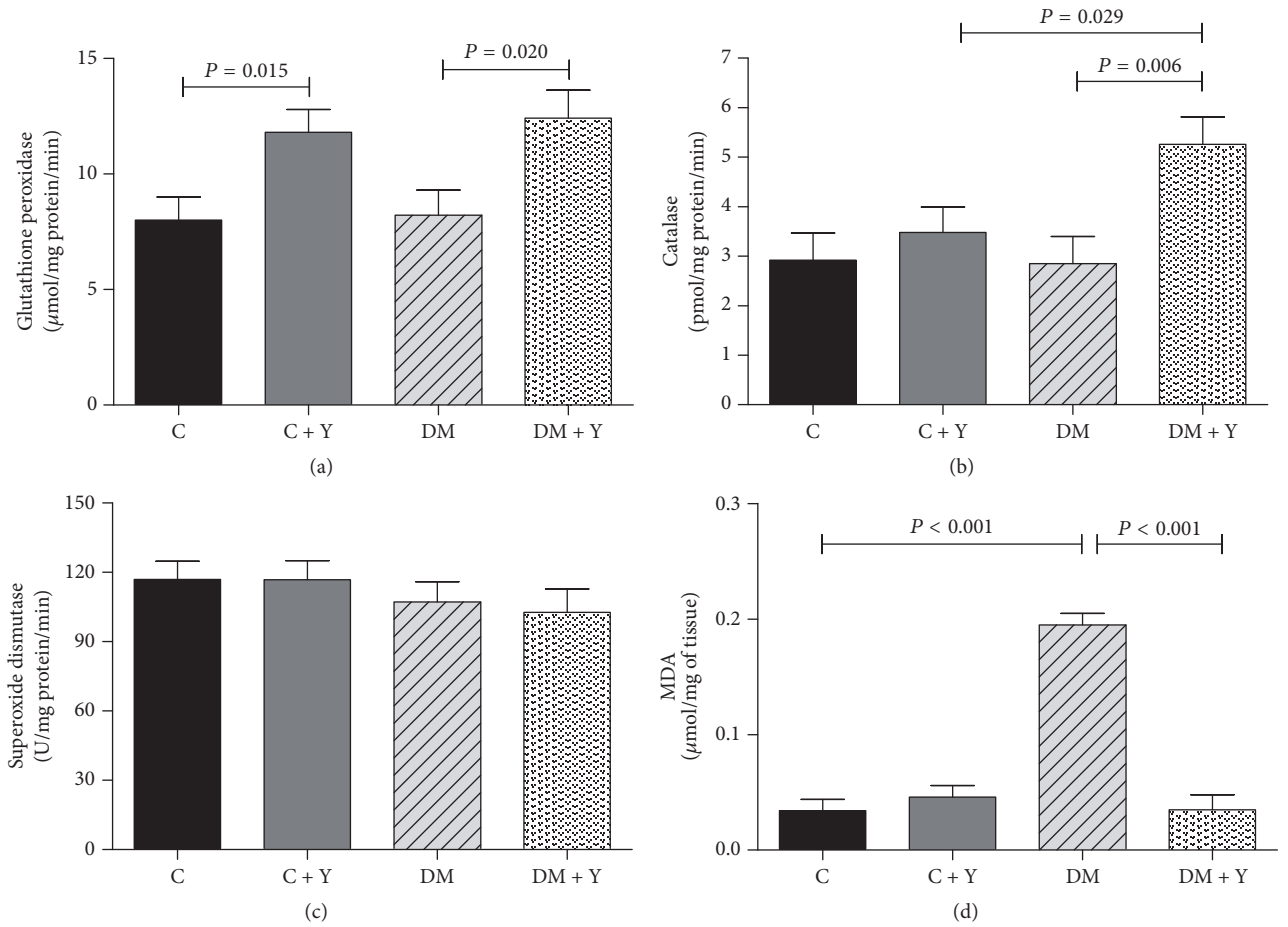

Figure 2: (a) Catalase (CAT) activity; (b) superoxide dismutase (SOD) activity; (c) glutathione peroxidase (GPx) activity; (d) MDA concentration in soleus of the different experimental groups. C (control group): normal rats; C + Y: normal rats receiving HEYL; DM: diabetic rats; and DM + Y: diabetics rats receiving HEYL. The results are expressed as the mean ± SEM.

activity among the groups (Figure 3(b)), whereas the GPx (Figure 3(a)) was higher in C + Y and DM + Y. No significant difference was found for SOD (Figure 3(c)) among the groups.

3.4. Hydrophilic Antioxidant Capacity (HAC) and IL-6 in Serum and Soleus Muscle. There were no significant differences for plasma IL-6 (Figure 2(c)). Plasma HAC was higher in C + Y when compared to C group, although, when evaluated in soleus muscle, a 2.2-fold increase of IL-6, with a 29.1% decrease of HAC, was observed in DM group compared to C group (Figure 2). HEYL promoted decrease of IL-6 and increase of HAC in DM + Y group when compared to untreated group.

4. Discussion

Streptozotocin (STZ) is a widely used chemical for the induction of experimental diabetes [20]. Type 1 diabetes can be induced in rodents by a single STZ injection [21]. All these STZ-induced diabetic animal models have been useful in elucidating the mechanisms of diabetic pathogenesis and in screening natural products and pharmacological agents that are potentially capable of lowering blood glucose levels [22] and attenuating the oxidative stress and inflammation. Under our experimental conditions, Wistar rats treated with a single dose of 40 mg STZ/kg bw underwent a marked hyperglycemia (395–416 mg/dL).

The administration of HEYL (100 mg/kg/d) in diabetic animals reduced serum glucose. These results are in agreement with Aybar et al. (2001) [23] and Genta et al. (2010) [24] studies, in which different extracts preparations and doses of Yacon, administered orally, reduced glycemia in STZ-induced diabetic rats, although Raga et al. (2010) [25] demonstrated that a dose of 100 mg/kg bw of Yacon tea presents more potential activity on glycemic control. The rate of blood glucose reduction in the present study occurred in synergy with the normally functioning pancreatic cells. The DM group without treatment has the lowest concentrations of insulin in plasma. The HEYL promoted a slight increase of insulin concentrations (Figure 1(c)), even without significant difference when compared to untreated DM group, and HOMA-BETA (Figure 1(d)), suggesting regeneration of functional β-cells.

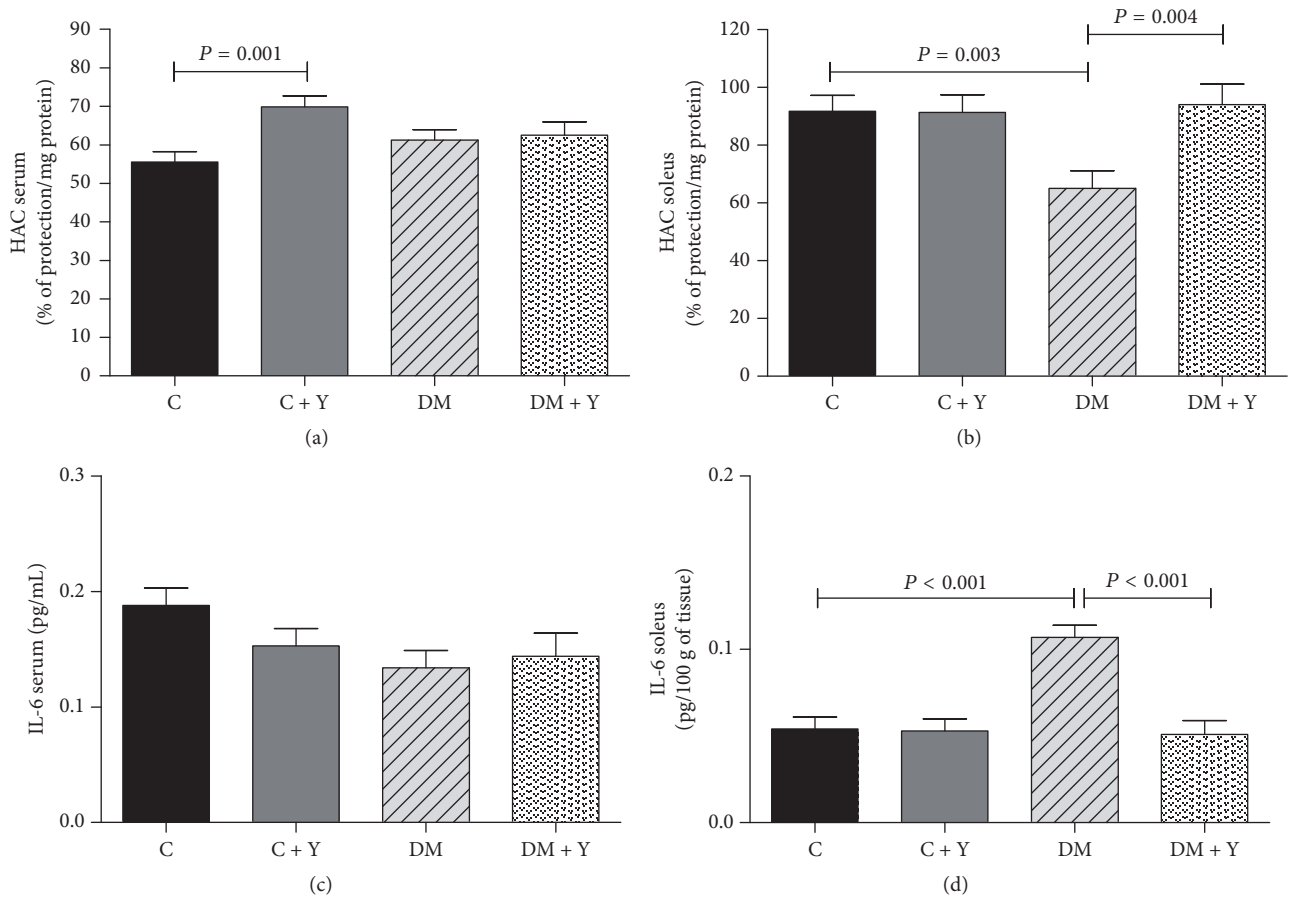

Figure 3: (a) Serum hydrophilic antioxidant capacity (HAC); (b) soleus HAC; (c) serum interleukin-6 (IL-6); (d) soleus IL-6 of the different experimental groups. C (control group): normal rats; C + Y: normal rats receiving HEYL; DM: diabetic rats; and DM + Y: diabetics rats receiving HEYL. The results are expressed as the mean ± SEM.

It is well known that hyperglycemia is the major cause of diabetic complications. Oxidative stress is one of the potential mechanisms by which hyperglycemia can result in diabetic complications [26]. Improvement of glycemic control that achieves near-normoglycemia can decrease the development and progression of its complications [25]. Regeneration or protection of pancreatic cells that were partially destroyed by STZ with increase of insulin concentrations in plasma and probably increase in the peripheral utilization of glucose could be factors that can explain the significant decrease of fasting blood glucose in the present study [27]. Additionally, some phytochemicals such as flavonoids and polyphenols have been found to be effective due to some other extrapancreatic mechanisms [28]. Further studies are in progress to establish the precise mechanism involved in the antihyperglycemic effect of HEYL.

It has been shown that the solvent used in the preparation of plant extracts can affect positively or negatively the biologically active principles of these plants [8]. Baroni et al. (2008) [29] showed that the hydroethanolic extract of Yacon leaves was the best extraction to promote reduction of glycemia in diabetic and nondiabetic animals. Also, the polyphenolics in Yacon leaves may regulate the free radical activity of STZ diabetes induction [10] and the pathogenesis of diabetes [30]. Plants rich in phenolic compounds have potential hypoglycemic effects [31, 32]. Ferulic acid, p-coumaric acid, caffeic acid, chlorogenic acid, protocatechuic acid, and quercetin were the highest compounds found in the extract. Jung et al. (2011) [33] found that low doses of onion peel hydroethanolic extract ameliorate hyperglycemia and insulin resistance in high-fat diet/STZ-induced diabetic rats in 8 weeks of treatment. Additionally, Pereira Braga et al. (2013) [34] described that isolated quercetin promotes glucose regulation and decrease of lipid peroxidation in diabetic animals. Caffeic and chlorogenic acids are known for their antioxidant and free radical scavenging properties [35]. Recently, caffeic acid in particular has been associated with reduced blood glucose [36]. Baroni et al. (2016) [37] showed that the phytochemical analysis of the hydroethanolic extract of Yacon identified the presence of phenolic compounds such as caffeic acid, ferulic acid, gallic acid, and chlorogenic acid, corroborating with the present study. The phytochemical profile may explain the antioxidant and antihyperglycemic activities noted in our study.

It is known that the pathogenesis of DM and its complications are associated with the overproduction of ROS and

depletion of the endogenous antioxidant system, leading to oxidative stress [38]. It is also known that skeletal muscle is a primary tissue in the response to metabolic alteration inducing physiopathological stimulus. Several signaling pathways in striated muscle can be activated by an increase in ROS production [39]. HEYL increased the activity of catalase and GPx (Figure 3) in the soleus muscle is likely attributed to improvement in glucose oxidation or direct modulation of antioxidant enzymes. Some reports suggest that oxidative stress is a key player to diabetic complications, which may be associated with alterations in the metabolism [40, 41]. In addition, it has been reported that STZ induces severe oxidative stress in diabetic animals caused by the peroxidation of polyunsaturated fatty acids, leading to the formation of MDA as by-products of lipid peroxidation [42]. Excessive lipid peroxidation can readily attack the polyunsaturated fatty acids of the lipid membrane, which in turn can disrupt the structure of biological membranes and produce toxic metabolites such as malondialdehyde [43]. MDA is often used as a marker of oxidative damage [44, 45]. In summary, excess ROS overwhelm antioxidant defenses, leading to oxidative stress.

No significant alterations were found in the plasma of diabetic animals for IL-6 (Figure 2(c)) when compared with controls. Although we did not observe changes in oxidative stress and inflammation markers when they were systemically evaluated after Yacon treatment, the leaves efficiently reduced metabolic markers, such as hyperglycemia, and oxidative/inflammation stress in soleus muscle. In addition, we observed that HAC decreased, while MDA and IL-6 increased in the soleus of diabetic animals (Figure 3), showing the oxidative stress and inflammation in this disorder.

The antioxidant activities of various vegetables, fruits, and plants are mainly attributed to their content of phenolic compounds [46]. The radical scavenging activity of polyphenols depends on the molecular structure and the substitution pattern of the hydroxyl groups, the availability of phenolic hydrogens, and the possibility of stabilization of the resulting phenoxyl radicals via hydrogen donation or by expanded electron delocalization [47]. This radical scavenging ability of extracts could be related to the nature of phenolics, thus contributing to their election transfer/hydrogen donating system.

In the present study, for the first time, the significant increase of the antioxidant status (HAC) and endogenous antioxidant activities (GPx and CAT) and decrease of markers of lipid peroxidation (MDA) and proinflammatory cytokine (IL-6) in soleus muscle in diabetic rats treated with HEYL suggest the antioxidant and anti-inflammatory activity of Yacon extract in this tissue. These results indicate that Yacon leaves have significant effects on scavenging free radicals, promoting decrease of oxidative stress under diabetic conditions.

Although the HEYL promoted several benefits on STZ-induced diabetic model, especially those regarding the glucose homeostasis and antioxidant activities, this study has limitations. The precise mechanisms by which HEYL promoted antihyperglycemic activity and increase of insulin concentrations have not been evaluated, even if they are hypothetically attributed to regeneration/preservation of pancreatic beta-cells. But further studies are in progress to investigate the precise mechanism/pathways involved.

STZ administration induces hyperglycemia and increases MDA and IL-6 in soleus muscle, toxic intermediates in the development of oxidative stress and inflammation in diabetes. Moreover, experimental diabetes decreases the capacity of antioxidant defenses in soleus muscle. In summary, these results demonstrate hyperglycemia-induced oxidative stress in skeletal muscle of diabetic rats. In conclusion, the hydroethanolic extract from *S. sonchifolius* leaves (HEYL) protects against hyperglycemia, oxidative stress, and inflammation in skeletal muscle and also promotes increase of serum insulin concentrations in STZ-induced diabetic model in rats. These findings provide information that can guide future studies aimed at finding therapeutic alternatives for diabetic complications.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript; Klinsmann Carolo dos Santos and Camila Renata Corrêa elaborated the experimental design; Klinsmann Carolo dos Santos, Bianca Guerra Bueno, Lahis Fernandes Bincoleto, Fabiane Valentini Francisqueti, Lilian Xavier da Silva, Ana Cláudia de Melo Stevanato Nakamune, Lahis Fernandes Bincoleto, and Mariana Gobbo Braz conducted the experiments; Klinsmann Carolo dos Santos, Ana Cláudia de Melo Stevanato Nakamune, C.-Y. Oliver Chen, Jeffrey B. Blumberg, and Camila Renata Corrêa analyzed the data; Klinsmann Carolo dos Santos, Lahis Fernandes Bincoleto, Mariana Gobbo Braz, C.-Y. Oliver Chen, Jeffrey B. Blumberg, and Camila Renata Corrêa revised the manuscript; Klinsmann Carolo dos Santos, C.-Y. Oliver Chen, Jeffrey B. Blumberg, and Camila Renata Corrêa wrote the manuscript.

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Conclusão

Conclusão

O modelo experimental utilizado (estreptozotocina) para indução do diabetes promoveu hiperglicemia, por levar a destruição das células beta pancreáticas, com perda da arquitetura das ilhotas e, consequentemente, diminuição da síntese e liberação de insulina, além de levar ao aumento de marcadores de estresse oxidativo séricos e teciduais, diminuindo a capacidade antioxidante no músculo sóleo e cardíaco, promovendo alterações metabólicas e desorganização celular e fibrose (depósito de colágeno) no tecido cardíaco. Resumidamente, animais diabéticos não tratado apresentaram danos oxidativos no músculo esquelético e indicadores de cardiomiopatia diabética. Interessantemente, o tratamento com extrato bruto das folhas de Yacon promoveu melhora destas alterações metabólicas, reduzindo os níveis de glicose e aumentando os níveis de insulina séricos. Estes dados podem ser atribuídos à preservação/proteção das células beta pancreáticas que foram parcialmente destruídas. Consequentemente, os danos oxidativos gerados pela disfunção metabólica gerada na condição diabética foram restaurados. Os efeitos benéficos observados podem estar associados a capacidade antioxidante do extrato. Nossos achados podem guiar futuros estudos com objetivo de elucidar e buscar terapias alternativas e complementares que possam auxiliar no tratamento do diabetes tipo 1 e suas complicações.