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Cucurbita ficifolia Bouché Regulates the Metabolism of Carbohydrates and Lipids in Liver by Activation of PPARα without Affectation on PPARγ *in Vivo* and *in Vitro*

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Abstract

Diabetes mellitus control in Mexico is practiced by using antidiabetic agents and, by empirical way using medicinal plants. *Cucurbita ficifolia* (*C. ficifolia*) has been attributed with hypoglycemic, hypotriglyceridemic, and anti-inflammatory effects, and D-chiro-inositol (DCI), 4-hydroxybenzoic acid, and β-sitosterol were proposed as active principles. The last two compounds were suggested as activators of two transcription factors, PPARα and PPARγ, in C2C12 myocytes, which participate in β-oxidation of fatty acids and insulin sensitivity. However, the involvement of the hepatocytic and adipocytic PPARs in the effects of *C. ficifolia* has not yet been explored. This research aimed to determine the effects of *C. ficifolia* on PPARα, PPARγ, and inflammatory cytokines in streptozotocin (STZ)-induced diabetes mice, HepG2 hepatocytes and 3T3-L1 adipocytes, implicating two additional cell types associated with metabolism of carbohydrates and lipids. STZ-induced diabetes mice received *C. ficifolia* (200 mg/kg/day) for 30 days, measuring serum cytokines (TNF-α and IL-6). RNA was extracted from liver. Besides, HepG2 and 3T3-L1 cells were incubated (24 h) with *C. ficifolia* (0.078 mM DCI), using pioglitazone or fenofibrate as controls. RNA was also extracted from cells and PCR in real-time was performed to determinate PPARα and PPARγ expression. In diabetic animals, *C. ficifolia* decreased glycemia and body weight, decreasing the expression level of TNF-α and IL-6. In addition, *C. ficifolia* increased PPARα expression in liver of diabetic animals, in HepG2 and 3T3-L1 cells; PPARγ

expression only significantly increased in HepG2 cells. The data suggest that the effects on the glycemia and lipids of *C. ficifolia* and its anti-inflammatory effects imply, besides skeletal muscle cells, hepatic and adipocytic PPARα activation, without affectation on PPARγ. PPARs regulation by C. ficifolia may improve the metabolic dysfunctions associated with metabolic disease, controlling the intake, activation, and oxidation of fatty acids and lipid storage.

Keywords

Cucurbita ficifolia, Diabetes Mellitus, Hypoglycemic Plants, Anti-Inflammatory Plants, PPARs, PPARα

1. Introduction

Cucurbita ficifolia Bouché (Cucurbitaceae) is an annual plant whose edible fruits have been attributed with medicinal properties. Several experimental and clinical studies have validated its biological effects. The hypoglycemic effect of the aqueous extract of the fruit was demonstrated in healthy and diabetic rabbits, mice, and rats, as well as in human diabetic patients [1] [2]. Then, it was reported its secretagogue action of insulin in RINm5F cells, which depends on the influx of Ca^{2+} from endoplasmic reticulum, an independent mechanism of sulphonylureas mediated by K^+ channels dependent on ATP [3] [4]. Furthermore, a liver histological analysis in alloxan-induced diabetes mice showed an accumulation of glycogen mediated by an increase in glycogen synthase and a decrease in glycogen phosphorylase [5]. Interestingly, the histological architecture evidenced a liver-protective effect due to the extract [5].

Since type 2 diabetes mellitus patients present situations of oxidative stress, dyslipidemia, and chronic inflammation, the antioxidant, and anti-inflammatory effects of fruit *C. ficifolia* aqueous extract were studied in streptozotocin (STZ)-induced diabetes mice [6] [7] and 3T3-L1 adipocytes [8]. In consequence, fruit *C. ficifolia* aqueous extract was proposed as a cytokine's inflammatory modulator, which was confirmed in monosodium glutamate-induced obesity mice, increasing the expression of IFN-γ and IL-10, whereas decreasing the expression of IL-6, TNF-α, and resistin, without changes in adiponectin's expression [8] [9].

Concerning the phytochemistry of *C. ficifolia* fruit, in 2006, Xia and Wang proposed D-chiro-inositol (DCI) as one of the responsible principles of the hypoglycemic effect of *C. ficifolia* [10]. Other identified secondary metabolites in the extracts of the fruit were phytosterols and fatty acids: β-sitosterol was the most abundant component, followed by 1,3-dimethyl-3-hydroxy-5-methoxyoxindole, 2H-1,4-benzoxazin-3(4H)-one, 2-butyl-4-hydroxy, 3-[(3,5-dimethoxybenxoyl)hydrazono]-N-(2-methoxyethyl)butyramide, 4-cyano-4-hydroxy-3-methyl-2-phenylpiperidine, stigmast-7-en-3-ol, benzoic acid, undecyl ester, stigmasta-5,24(28)-dien-3-ol, and stigmasta-5,22-dien-ol [11]. Also identified were the 4-hydroxybenzoic acid, hydroxyphenyl acetic acid, gallic acid, salicin, catechin, p-coumaric

acid, and cinnamic acid. In these studies, β-sitosterol and 4-hydroxybenzoic acid were pharmacologically evaluated [5] [11]. Both compounds increased insulin secretion in RINm5F cells, probably mediated by the receptors G-protein coupled receptor 40 (GPR40) and increased the activation of one member of the family of peroxisome proliferator-activated receptors (PPARs), PPARγ in C2C12 myocytes [11].

Although the aqueous extract from fruit of *C. ficifolia* has hypoglycemic, hypolipidemic, antioxidant, and anti-inflammatory actions, little is known about its effects on the energetic balance regulated by the peroxisome proliferator-activated receptors (PPARs), which play an essential role in all these actions in key metabolic tissue as liver and adipose tissue. PPARs are transcription factors, members of the nuclear hormone receptor superfamily that regulate carbohydrates and lipids' metabolism [12] [13] Three PPARs subtypes have been described (α, γ, and β/Δ), which possess differential tissue distribution and selective ligands [14]. PPARα is expressed notably in hepatocytes, whereas PPARγ is expressed mainly in adipocytes; PPAR β/Δ is more ubiquitous and is mainly expressed in adipose tissue and skeletal muscle.

PPARα activation in the liver plays an essential role in controlling fatty acid transport, β-oxidation, and the expression of proteins, such as long-chain-fatty-acid-CoA ligase 1 (ACSL-1), also participating in the inflammatory response [14]. In diabetes, PPARα in the liver is deregulated, contributing to hyperglycemia and accumulating fatty acids, inflammation, and fibrosis [14]. PPARγ activation in adipocytes increases GLUT-4 and fat accumulation, thereby increasing adiponectin and decreasing resistin and TNF-α expression, enhancing insulin sensitivity and reducing glycemia [14] PPARγ also regulates immunity and inflammation through adipokines, particularly adiponectin, whose secretion occurs in adipose tissue and skeletal muscle [15].

Since, in different experimental conditions, the aqueous extract from fruit of *C. ficifolia* has shown hypoglycemic, hypolipidemic, and anti-inflammatory actions [6] [7] [8] [9], the present research aimed to determine if this extract regulates these effects by activation of PPARα and PPARγ in liver and adipocyte tissue using approaching studies *in vivo* (STZ-induced-diabetes mice) and *in vitro* (HepG2 and 3T3-L1 cells).

2. Material and Methods

2.1. Plant Material

Fresh mature fruits of *C. ficifolia* (18 - 20 cm of diameter) were gathered from the Acolman Municipality, Estado de Mexico, in April and May. This material was identified by taxonomic keys and was compared with voucher specimen No. 11119 from the Medicinal Plant Herbarium of the Mexican Institute of Social Security at Mexico City (Herbarium IMSS-M). The seedless endocarp was dried at room temperature and ground using 2-mm mesh in a Model 4 Wiley electric mill.

2.2. Preparation of Aqueous Extract from *C. ficifolia* Fruit

The aqueous extract was obtained following the methodology of previous studies [2]. The ground material (100 g) from the *C. ficifolia* fruit was extracted with 300 mL of water for 24 h, at ambient temperature. This extract was filtered and freeze-dried, resulting in an aqueous extract yielding 35%.

2.3. Quantitative Analysis of D-Chiro-Inositol in the Aqueous Extract of *C. ficifolia*

An isocratic acetonitrile-methanol (8:2) mixture was used for elution over a LiChrospher 100 Å NH_2 column (5 μm, 4 × 250 mm). D-chiro-inositol was quantified using high-performance liquid chromatography (HPLC; Waters 2695 separation module) with a Waters 2697 Index Refractive Detector (Waters Milford, MA, USA). The concentration of D-chiro-inositol, which had a retention time of 8.6 min, was determined upon injection of the extract (20 μL/2mg/mL). The resulting standard curve was linear (R^2 = 0.99); the aqueous extract of *C. ficifolia* contained 3.32 mg of D-chiro-inositol/g of extract.

2.4. Experimental Animals

Male CD-1 mice (30 - 35 g) were obtained from the Laboratory Animal Center of the Metropolitan Autonomous University. They were maintained with rodent food (Harlan Laboratories, Indianapolis, USA) and water ad libitum under a 12 h light/dark cycle. All rodent procedures followed International Rules for the Care and Use of Laboratory Animals, in agreement with the Mexican Official Norm (NOM-062-ZOO-1999, revised 2001).

2.5. Evaluation of the Energetic Balance and Anti-Inflammatory Effect of the Aqueous Extract from Fruit of *C. ficifolia*

Considering that the aqueous extract from fruit of *C. ficifolia* can be used as prophylactic and curative treatments, we considered both stages in the experimental design. Normal mice were divided into four groups of 6 animals each. Groups 1 and 2 received saline solution (4 mL/kg/day), Group 3 received aqueous extract of *C. ficifolia* (200 mg/kg/day), and Group 4 pioglitazone (45 mg/kg/day), an insulin-sensitizer that activates PPARγ [16] or, in its case fenofibrate (100 μM) as a PPARα activator. The dose of 200 mg/kg/day of the aqueous extract of *C. ficifolia* used in the *in vivo* experiment was chosen of previous studies [5] [9] [11]. These agents were used as potential preventive treatments, which were given by gavage for 15 days. Afterward, Groups 2, 3, and 4 received a single intraperitoneal administration of 137 mg/kg streptozotocin (STZ) dissolved in citrate buffer (0.1 M, pH = 4.5). Group 1 received citrate buffer only. All treatments were continued for the other 30 days after administration of STZ.

2.6. Biochemical Parameters and Cytokine Quantification

Body weight, triglycerides, and glycemia were measured throughout the experi-

ment. The biomarkers of inflammation were quantified at the end of the treatments. Glycemia was determined in blood samples from a puncture of the tail vein and was quantified on the 15th day in normal mice with free access to water and food and on day 45 in fasted animals (12 h without food) using the hydrogenase method (Roche Diagnostics). On day 45, quantification of triglycerides was performed in Reflotron System (Bayer), using blood samples from the ocular orbital sinus of animals anesthetized with pentobarbital (200 mg/kg). At the end of the study (30 days after STZ administration), additional blood samples were obtained from the ocular orbital sinus for cytokine analysis. Serum cytokine levels were quantified using enzyme-linked immunosorbent assay (ELISA) kits purchased, IL-6 was analyzed from Pierce Protein Research Products (Thermo Fisher Scientific, Illinois, USA) and TNF-α from R & D Systems (Minneapolis, USA). The RNA was isolated from the liver by using a Trizol reagent (Thermo-Fisher Scientific, MA, USA). The RNA (1 μg) was electrophoresed using a 1% agarose gel, stained with ethidium bromide, and visualized using the Image Gel-Logic 212 Pro (Kodak/Carestream, Rochester, NY, USA). To confirm the integrity of RNA, absorbance at 260 and 280 nm was measured for each RNA sample; the OD ratio (260/280 nm) was 1.9 ± 0.2, consistent with the absence of protein contamination. Two major ribosomal bands (28S and 18S rRNA) without RNA degradation were detected (data not shown).

2.7. HepG2 and 3T3-L1 Cells Culture

HepG2 cellular line of human hepatoblastoma was acquired from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were maintained in Williams medium (Sigma, St Louis, MO) supplemented with fetal bovine serum at 8% (FBS, Hy-Clone, Logan, UT, USA), 100 U/mL penicillin, and 100 μL streptomycin (Micro lab). Cells were grown in plastic bottles in sterile conditions (Corning, Tewksbury, MA, USA), changing the medium each 48 h. Trypsinized cells (trypsin 0.25% and EDTA 0.2 M (Sigma)) were diluted and cultured weekly 1:3 in culture bottles. Culture cells were incubated in 5% CO_2, 95% moisture, and a temperature of 37˚C. All experiments were performed in the logarithmic phase of cell growth. HepG2 cells were treated for 24 h with the aqueous extract of *C. ficifolia* (containing 0.078 mM of DCI), using as positive controls pioglitazone or fenofibrate. After 24 h of treatment, RNA was extracted from culture cells by the Trizol method. From extracted RNA, the cDNA and PCR in real-time were performed.

The 3T3-L1 cells of mice fibroblasts were cultures in Eagle modified medium Dulbecco (DMEM, GIBCO, Grand Island, NY, USA) with 10% calf serum and glucose 25.5 mM, in culture bottles of 75 cm at 37˚C, 5% de CO_2 and 95% humidity. These cells in preadipocyte conditions were differentiated into adipocytes. The 3T3-L1 mice fibroblasts (8×10^5 cells/well) were cultivated at the confluence in the same conditions (37˚C, 5% CO_2), glucose 25.5 mM, sodium pyruvate 1 mM, glutamine 2 mM, non-essential amino acids (0.1 mM), gentamicin

(20 μg/mL), and complemented with 10% of FBS. After two days of confluence (day 0), the differentiation was induced with metil-hydroxybutylxanthine (MIX, 0.5 mM), dexamethasone (DX, 0.25 μM), and insulin (5 μg/mL) in DMEM with 10% FBS. On the second day, the medium was changed and added with insulin (5 μg/mL), free of MIX and DX, incubating for another two days. From the fourth day, medium free of insulin was replaced each 48 h. Cells were used after eight days of differentiation [17].

The 3T3-L1 cells were incubated for 24 h with the aqueous extract of *C. ficifolia* (containing 0.78 mM of DCI) and the positive controls (10 μM pioglitazone and 100 μM fenofibrate). After 24 h of treatment, RNA was extracted from the cells by the Trizol method. From RNA was prepared, the cDNA and PCR in real-time were performed.

2.8. mRNA Expression of PPARγ, PPARα, TNF-α, and IL-6

Two micrograms of total RNA were reverse transcribed using the ImProm II reverse transcription system (Promega, Madison, WI, USA). The reaction (20 μL) was incubated in a thermocycler Select Cycler (BioProducts, West Palm Beach, FL, USA), following the next cycle program: incubation for 5 min at 25˚C, extension at 42˚C for 55 min, the enzyme was inactivated at 70˚C for 15 min, and lastly, the samples cooled to 4˚C for 5 min. Then, cDNAs were amplified with SYBR Green master mix (Roche Molecular Biochemicals, Mannheim Germany) containing 0.5 mM of customizing primers (**Table 1**) for TNF-α, IL-6, PPARγ, PPARα, GLUT-1, ACSL-1, FATP-1, GLUT-4, AdipoQ and, as internal controls, 36B4 and GADPDH, each one plus Fast Star Enzyme, PCR buffer and 3.5 mM $MgCl_2$ in a final volume of 10 μL. The reactions were measured in a rotor gene system. PCR was conducted using the following cycling conditions: enzyme pre-incubation during 10 min at 95˚C, 35 or 40 cycles denaturizing at 95˚C for 10 sec, a thermal ramp rate of 20˚C per sec; annealing at 61˚C for 7 sec with a thermal ramp rate of 20˚C per sec; amplification at 72˚C for 10 sec with a thermal ramp rate at 20˚C per sec. The threshold cycles (Ct) were measured in separate tubes and duplicates. The identity and purity of the amplified products were checked by electrophoresis on 2% agarose mini gels. Analysis of the melting curve carried out at the end of amplification under the following conditions: denaturation at 95˚C, with a thermal ramp rate of 20˚C per sec, re-annealing at 65˚C for 15 sec, with a thermal ramp rate of 20˚C per sec, and finally slowly denaturizing at 95˚C with a thermal ramp rate of 0.1˚C per sec. Each assay included a negative control for each gene to guarantee the quantity of the measurements. The abundance of mRNA encoding 36B4 normalized the abundance of cytokines mRNA. The ΔCt values were calculated in every sample for each gene of interest as follows: Ct interest—Ct reference with 36B4 or GADPDH as the reference gene (mRNA of reference remained stable throughout the experiments). Relative changes in the expression level of each gene (ΔΔCt) were calculated as ΔCt sample minus ΔCt reference and then presented as $2 - \Delta\Delta Ct$ [18].

Table 1. Genes and primers used in STZ-induced diabetes mice, HepG2 and 3T3-L1.

Genes liver mice	Primer forward	Primer reverse	Pb
36B4 Gene bank NM_007475.2	AAGCGCGTCCTGGCATTGTCT	CCGCAGGGGCAGCAGTGGT	135
IL-6 Gene bank NM_031168.1	TTCCATCCAGTTGCCTTCTT	CAGAATTGCCATTGCACAAC	129
PPAR-γ Gene bank NM_011146.1	CCAGAGTCTGCTGATCTGCG	GCCACCTCTTTGCTCTGCTC	217
PPARα Gene bank NM_011144	ATGCCAGTACTGCCGTTTTC	GGCCTTGACCTTGTTCATGT	220
TNFα Gene bank NM_013693.2	ACTTGGTGGTTTGCTACGAC	CCTCCCTGTCATCAGTTCTA	102
Genes HepG2	**Primer forward**	**Primer reverse**	**Pb**
GADPH Gene Bank AF261085.1	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG	238
PPAR-α Gene BankNM_001001928.2	GTTTGAGGGGGTAACAGCAA	GCTAACTGCAGAGGGTGAGG	247
PPAR-γ Gene Bank NM_138712.3	GCTGTGCAGGAGATCACAGA	GGGCTCCATAAAGTCACCAA	225
GLUT-1 Gene Bank NM_006516.2	TCACTGTGCTCCTGGTTCTG	CCTGTGCTCCTGAGAGATCC	233
ACSL-1 Gene Bank NM_001995.2	CCAGAAGGGCTTCAAGACTG	GCCTTCTCTGGCTTGTCAAC	204
FATP-1 Gene Bank NM_198580.1	CCACTTGGATGTCACCACTG	GTGGGACCCTCCAGTAGACA	178
Genes 3T3-L1	**Primer forward**	**Primer reverse**	**Pb**
36B4 Gene Bank NM_007475.2	AAGCGCGTCCTGGCATTGTCT	CCGCAGGGGCAGCAGTGGT	135
PPAR-Δ Gene Bank NM_011145	TGGAGCTCGATGACAGTGAC	GTACTGGCTGTCAGGGTGGT	161
PPAR-γ Gene Bank NM_011146.1	CCAGAGTCTGCTGATCTGCG	GCCACCTCTTTGCTCTGCTC	217
GLUT-4 Gene Bank NM_009204.2	GATTCTGCTGCCCTTCTGTC	ATTGGACGCTCTCTCTCCAA	168
FATP-1 Gene Bank NM_011977.3	ACCAGTGTCCAGGGGTACAG	TGTCTCCCAGCTGACATGAG	174
Adipo Q Gene Bank NM_009605.4	GGCTCTGTGCTCCTCCATCT	AGAGTCGTTGACGTTATCTGCATAG	101

2.9. Statistical Analysis

Data are presented as the mean ± SEM. Statistical differences among the treat-

ments were determined by an analysis of variance using the Tukey-Kramer Multiple Comparisons post-hoc tests ($P < 0.05$). All statistics were computed using the NCSS 2000 software.

3. Results

3.1. Effect of the Aqueous Extract from Fruit of *C. ficifolia* on Glycemia, Body Weight, and Triglycerides in Normal Mice

Aqueous extract from *C. ficifolia* fruit administered during the first 15 days did not modify the glycemia of normal mice with free access to food nor prevented the initial STZ-induced hyperglycemia (data not shown). When given during the 30 days post STZ administration, the glycemia was found significatively diminished compared with diabetic control, exhibiting an effect like pioglitazone (Figure 1(a)). Body weight was decreased in diabetic mice without treatment ($P < 0.05$). *C. ficifolia* extract and none of the other treatments caused significant changes in body weight compared with diabetic control (Figure 1(b)). *C. ficifolia* extract and fenofibrate caused a significant decrease in triglycerides. Pioglitazone did not alter this parameter in STZ-induced diabetic mice (Figure 1(c)).

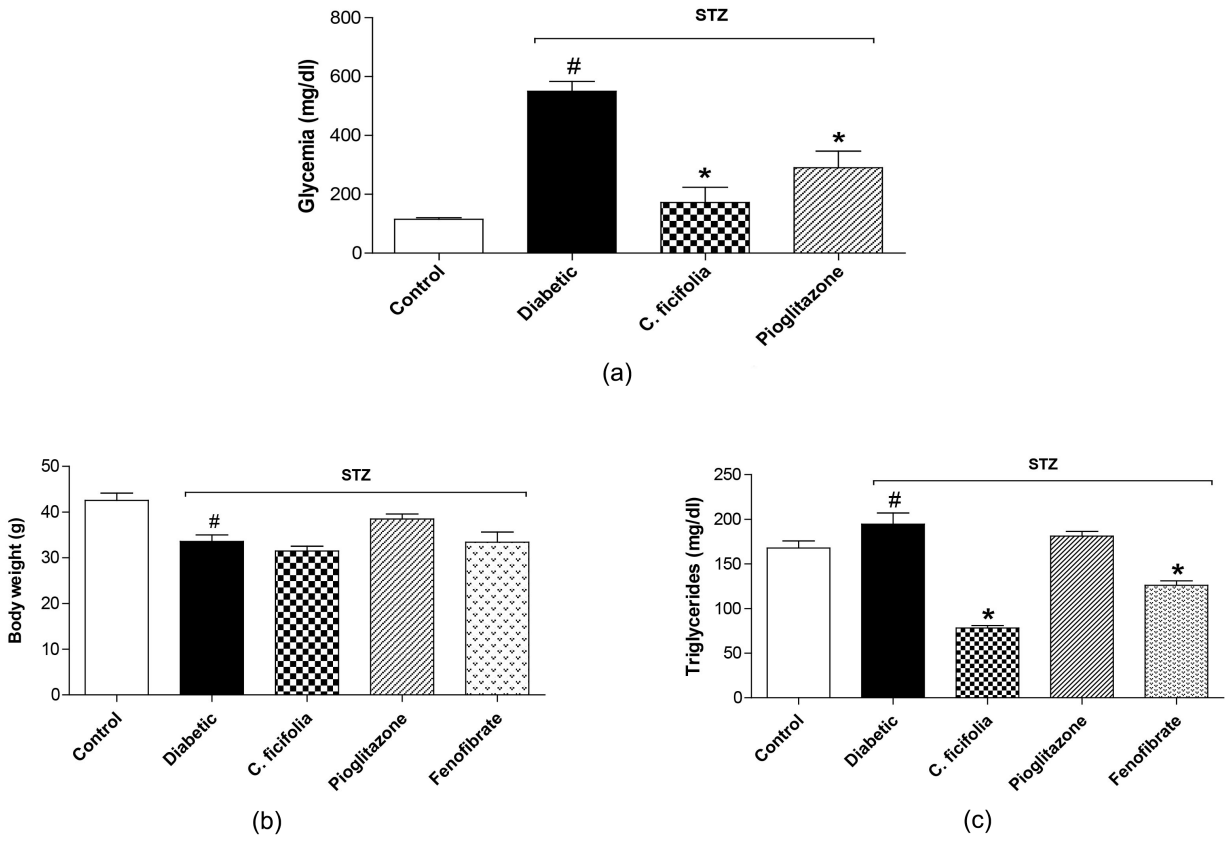

Figure 1. Effect of the daily administration of aqueous extract from fruit of *C. ficifolia* (30 days) on glycemia (a), body weight (b) and triglyceride levels (c) in STZ-induced diabetic mice. Mean ± S. E. M. (n = 6). #Statistically significant compared with normal control; *Statistically significant compared with diabetic control ($P < 0.05$). Control: normoglycemic mice; Diabetic: STZ hyperglycemic mice; *C. ficifolia*: STZ hyperglycemic mice treated with *C. ficifolia extract*; Pioglitazone: STZ hyperglycemic mice treated with pioglitazone.

3.2. Effect of the Aqueous Extract from Fruit of *C. ficifolia* on PPARα and PPARγ Gene Expression in Liver of STZ-Induced Diabetic Mice

PPARγ and PPARα gene expressions in diabetic mice were always lower than in normal mice (Figure 2(a) and Figure 2(b)). *C. ficifolia* extract did not affect PPARγ but significantly increased PPARα compared to diabetic control. Pioglitazone significantly increased the expression of PPARγ (Figure 2(a)), whereas fenofibrate significantly increase the expression of PPARα (Figure 2(b)).

3.3. Effect of the Aqueous Extract from Fruit of *C. ficifolia* on Expression and Serum Levels of IL-6 and TNF-α in STZ-Induced Diabetic Mice

The mRNA expression of IL-6 and TNF-α in the liver of STZ-induced diabetic mice was more prominent than in control normal mice. In serum, IL-6 and TNF-α were higher in diabetic mice than in control normal mice; however, just for TNF-α, the difference was statistically significant. *C. ficifolia* extract caused significant reduction in mRNA expression of both cytokines; however, only for TNF-α, the difference was significant (Figures 3(a)-(c)). *C. ficifolia* extract did not produce significant changes in serum levels of these cytokines (Figures 3(b)-(d). The treatment with pioglitazone significantly reduced TNF-α expression without significant changes in IL-6 expression or serum levels of IL-6 and TNF-α.

3.4. Effect of the Aqueous Extract from Fruit of *C. ficifolia* on PPARα, PPARγ in ACSL-1, GLUT-1, and FATP-1 in HepG2 Cells

Cellular viability was assessed in the HepG2 cell line with different doses. For evaluations, a safe concentration was established at 0.078 mM of DCI/g of extract from *C. ficifolia* fruit extract. *C. ficifolia* extract significantly increased

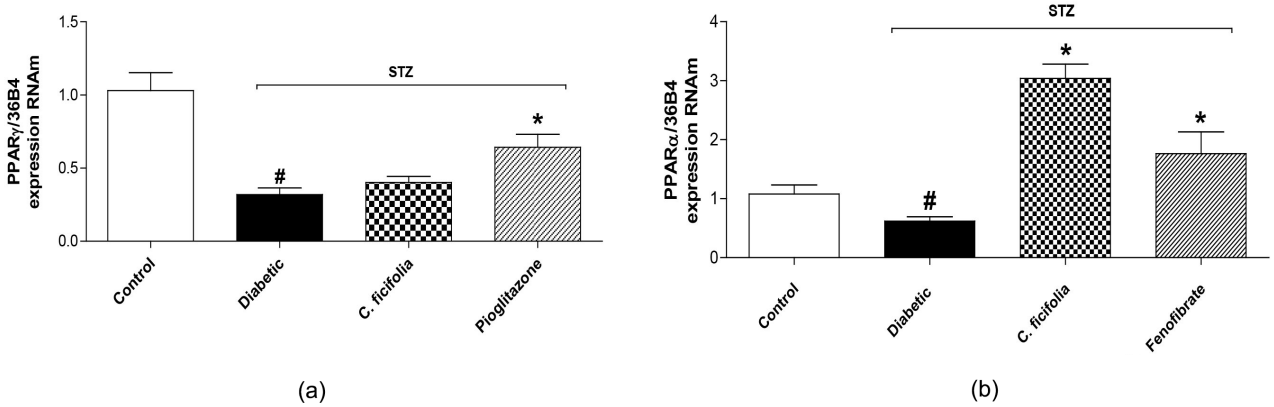

Figure 2. Effect of the daily administration of the aqueous extract from fruit of *C. ficifolia* (30 days) on PPARγ (a) and PPARα (b) expression. Mean ± S. E. M. (n = 6). #Statistically significant against normal control; *Statistically significant against diabetic control ($P < 0.05$). Control: normoglycemic mice; Diabetic: STZ hyperglycemic mice; *C. ficifolia*: STZ hyperglycemic mice treated with *C. ficifolia* extract; Pioglitazone: STZ hyperglycemic mice treated with pioglitazone.

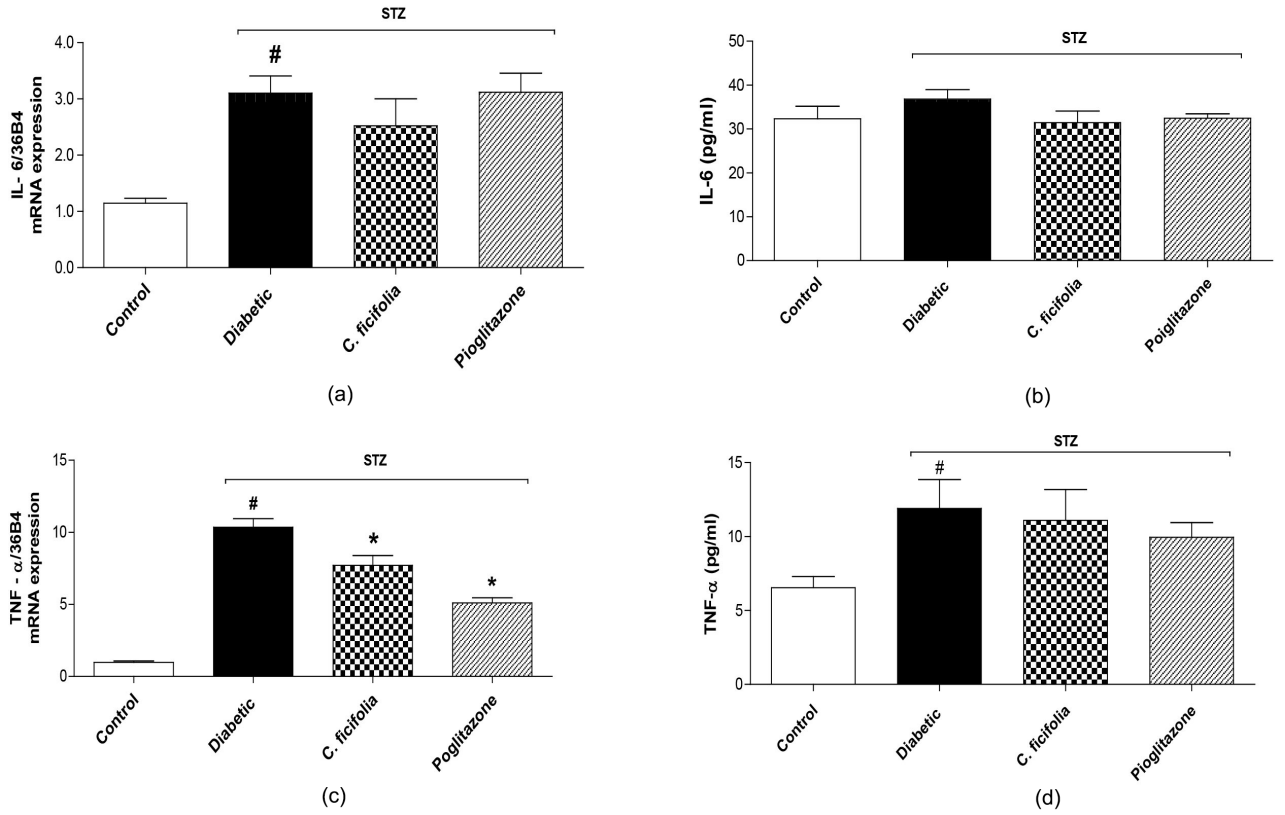

Figure 3. Effect of the daily administration of the aqueous extract from fruit of *C. ficifolia* (30 days) on the mRNA expression and serum level of IL-6 (a) and (b) and TNF-*α* (c) and (d). Mean ± S. E. M. (n = 6). #Statistically significant compared with normal control; *Statistically significant compared with diabetic control ($P < 0.05$). Control: normoglycemic mice; Diabetic: STZ hyperglycemic mice; *C. ficifolia*: STZ hyperglycemic mice treated with *C. ficifolia* extract; Pioglitazone: STZ hyperglycemic mice treated with pioglitazone.

PPAR*α*, PPAR*γ*, and GLUT-1 expression in HepG2 cells (Figures 4(a)-(c)). In contrast, it exhibited a non-significant reduction of FATP-1 and ACSL-1 against control (Figures 4(d) and Figure 4(e)). Fenofibrate significantly increased PPAR*α* and FATP-1 (Figure 4(a) and Figure 4(d)). Fenofibrate also exhibited a non-significant increase of ACSL-1 (Figure 4(e)). Pioglitazone significantly increased PPAR*γ* and GLUT-1 against control in HepG2 cells (Figure 4(b) and Figure 4(c)).

3.5. Effect of the Aqueous Extract from Fruit of *C. ficifolia* on PPAR*α*, PPAR*γ*, GLUT-4, FATP-1, and AdipoQ in 3T3-L1 Cells

In 3T3-L1 cells, *C. ficifolia* extract exhibited a non-significant increase of PPAR*α*, PPAR*γ* and AdipoQ (Figure 5(a) and Figure 5(b) and Figure 5(e)) and significantly increased GLUT-4 and FATP-1 against control (Figure 5(c) and Figure 5(d)). Pioglitazone exhibited a significant increase of PPAR*γ*, GLUT-4, and AdipoQ against control (Figure 5(b) and Figure 5(c) and Figure 5(e)). Fenofibrate exhibited a significant increase of PPAR*α* and FATP-1 against control (Figure 5(a) and Figure 5(d)).

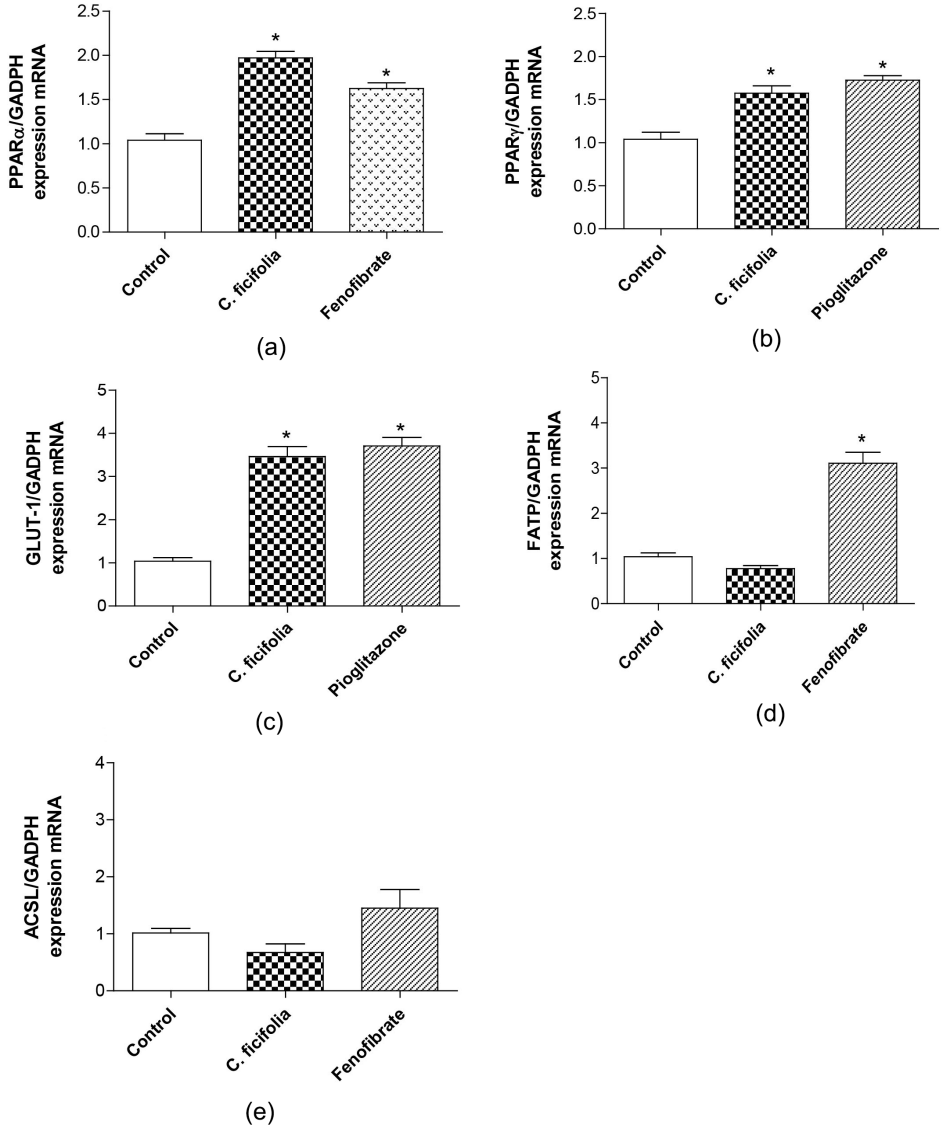

Figure 4. Effect of the aqueous extract from fruit of *C. ficifolia* (24 h of incubation) on mRNA expression of PPARα (a), PPARγ (b), ACSL-1 (c), GLUT-1 (d), and FATP-1 (e) in HepG2 cells. Mean ± S. E. M. (n = 6). *Statistically significant compared with diabetic control ($P < 0.05$).

4. Discussion

Diabetic and obese patients have alterations in energy balance, accumulating the energy consumed as fat and, consequently, present insulin resistance, hyperglycemia, dyslipidemia, oxidative stress, and chronic inflammation. These anomalies can be associated with deregulating the peroxisome proliferator-activated receptors (PPARs), which play an essential role in the energetic balance that depends on the energy uptake and consumption [19]. Since the aqueous extract from fruit of *C. ficifolia* has hypoglycemic, hypolipidemic, and anti-inflammatory actions, it was considered essential to study the regulatory capacity of this extract on PPARs in an *in vivo* model of STZ-induced diabetic mice and in two *in vitro* models using HepG2 hepatocytes and 3T3-L1adipocytes.

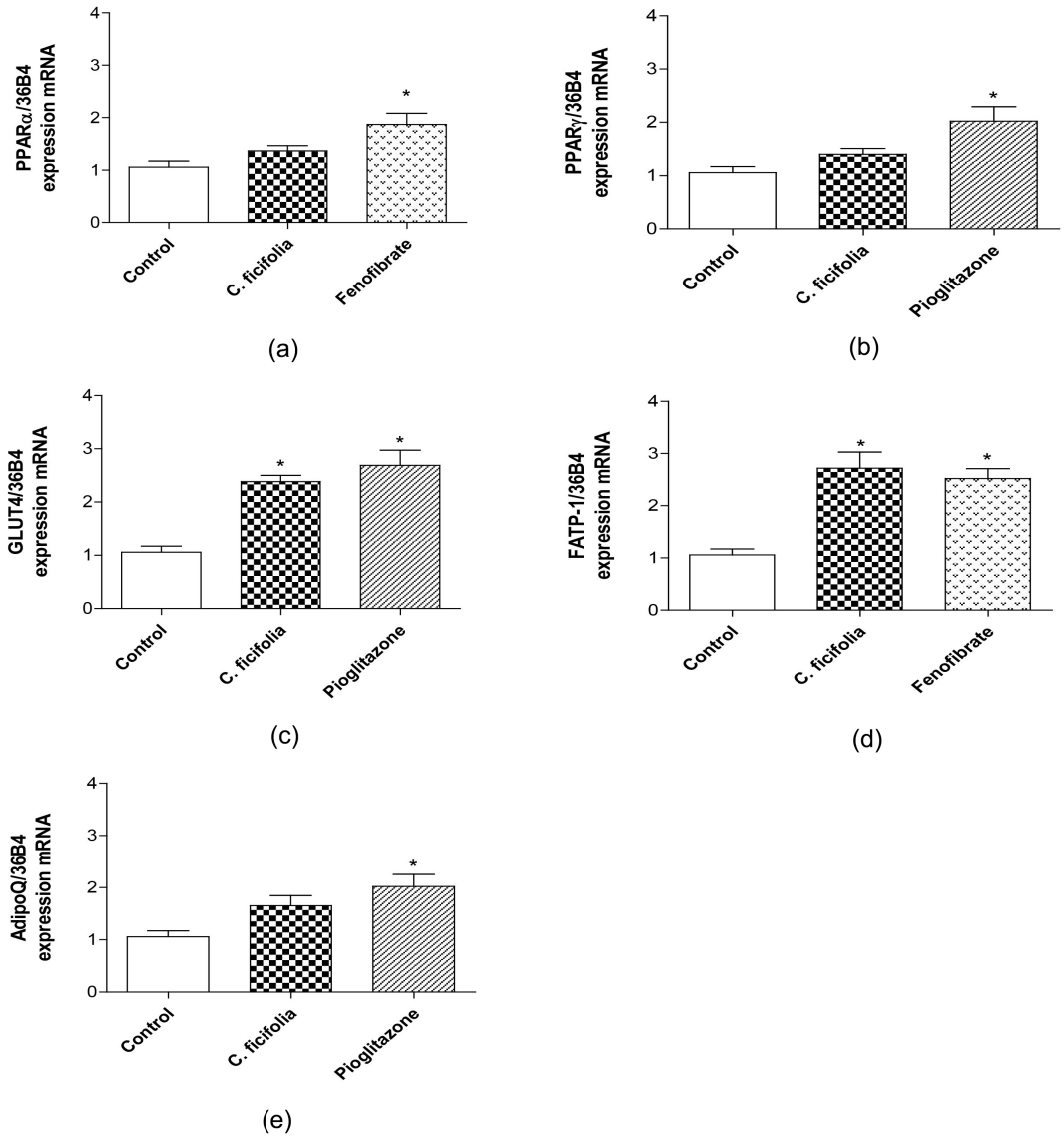

Figure 5. Effect of the aqueous extract from fruit of *C. ficifolia* (24 h of incubation) on mRNA expression of PPAR*γ*, GLUT-4 and FATP-1, and AdipoQ in 3T3-L1 cells. Mean ± S. E. M. (n = 6). *Statistically significant compared with diabetic control ($P < 0.05$).

In STZ-induced diabetic mice, the daily administration of *C. ficifolia* extract and pioglitazone for 30 days significantly reduced 2-fold glycemia and 1-fold triglyceridemia, without changes in body weight. The hypoglycaemic activity of *C. ficifolia* extract was reported previously in experimental models and humans [1] [2] [10] and our results are congruent with the previous data.

Loss of body weight is considered a typical condition in diabetic patients, which is associated with hyperglycemia and hypertriglyceridemia. Control diabetic mice showed weight loss; however, any treatment from the present study modified this parameter. In addition to reducing glucose, *C. ficifolia* extract also significantly decreased triglyceride levels in diabetic mice, as reported previously by [6] also in STZ-induced diabetes mice. In contrast, pioglitazone lowered

blood glucose but did not exhibit an effect on triglycerides. Pioglitazone is a thiazolidinedione (TZD) with PPARγ agonist action that improve insulin sensitivity. This action occurs by regulating gene expression of several proteins involved in the lipogenesis of adipocyte tissue [20] [21], causing fatty liver and hepatic steatosis, a condition considered one of the principal undesirable effects of TZDs.

PPARs are transcription factors composed of three isoforms, PPARα, PPARβ/Δ, and PPARγ. These factors regulate energetic balance by expressing several proteins involved in lipogenesis and lipolysis [16] [22]. *C. ficifolia* extract did not change PPARγ gene expression in the liver of STZ-induced diabetic mice, compared with diabetic control. Instead, it showed a significant reduction of 20% in TNF-α expression, an important inflammatory modulator. Besides, *C. ficifolia* extract significantly increased 2-fold PPARα, which is involved in the uptake, activation, and oxidation of fatty acids [16] [22]. These data in diabetic mice suggest that *C. ficifolia* extract propitiates a new energetic balance decreasing glucose and triglycerides and improving inflammatory conditions associated with decreased TNF-α expression. Pioglitazone increased PPARγ, PPARα, and TNF-α gene expression and reduced glycemia without modifying triglycerides. Since *C. ficifolia* extract showed the hypotriglyceridemic effect, this may represent an advantage against TZD agents, avoiding liver fat accumulation. However, the mechanisms implicated in these effects of *C. ficifolia* extract should be explored in further studies.

Results in STZ-diabetic mice also suggest that *C. ficifolia* extract can act like a PPARα agonist, modulating the glucose/lipids balance. [15] demonstrated that ob/ob mice and Zucker rats, subjected to PPAR-α agonist treatment, decreased body fat, glycemia, and insulin levels, promoting sensitive insulin ([23]. PPARα is stimulated by fibrates such as gemfibrozil, clofibrate, fenofibrate, and ciprofibrate, decreasing triglycerides and increasing high-density lipoproteins (HDL). In addition, PPARα promotes lipolysis and decreases very low-density lipoproteins (VLDL) [19]. Although some biomarkers of metabolism of carbohydrates in liver were measured in previous studies [5], other biomarkers of metabolism of carbohydrates and lipids must be further studied.

PPARα in the liver modulates gene expression of enzymes that participate in the mitochondrial and peroxisomal fat acids oxidation and ketogenesis, like 3-hidroxy-3-methiylglutaril-CoA synthetase 2 (HMGCS2), carnitine palmitoyl transferase A (CPT1A), carnitine palmitoyl transferase 2 (CPT2), enoyl CoA synthetase 1 peroxisomal (ECH1), as well as for the expression of genes that participate in the binding and activation of fat acids, like the liver fat acids binding protein 1(FABP1), and the isoforms 1 and 3 of long-chain-fatty-acid-CoA ligase (ACSL-1, ACSL-3) [14]. In HepG2 cells, *C. ficifolia* significantly increased PAPRα (1-fold), PPARγ (0.5-fold), and GLUT-1 (2-fold) gene expression and caused a non-significant decrease in ACSL-1 and FATP-1. Concerning PPARα, this result in HepG2 cells is congruent with the found in the liver of STZ-induced diabetic

mice. Hence the importance of PPARα regulation in lipid metabolism.

The ACSLs catalyze the first pass in lipid metabolism, modifying long-chain fatty acids in acyl CoA thioesters. Five isoforms in mammals have been described [24] [25]. The acetyl-CoA groups participate in anabolic and catabolic routes, participating in triglycerides synthesis and oxidation processes. The isoform ACSL-1 is highly expressed in the liver and adipose tissue. Therefore, expression of RNAm of Acsl-1 in these tissues is potentiated by the activation of PPARα, participating in the β-oxidation of fatty acids [26].

Fenofibrate, a PPARα agonist, promoted mRNA expression of PPARα and ACSL-1. In HepG2 cells, *C. ficifolia* increased PPARα expression without significantly modifying ACSL. These results are congruent with previous studies in liver ACSL-1 knock-out mice, in which was observed decrease of 50% in the total activity of the ACSLs and reductions of 25% to 35% in the content of long chain acetyl-CoA [24]. Besides, anabolic and catabolic routes may be altered in pathologies such as liver steatosis, hyperlipidemia, and insulin resistance [24]. The data also suggest that *C. ficifolia* extract may transcriptionally stimulate PPARα expression *in vivo* and *in vitro* without activating the pathways associated with ACSL-1 and FATP-1 in Hep-G2 cells submitted at 24 h de incubation. Whatever it is, further studies will be mandatory to perform studies to know the temporal curse variations in the expression and protein levels of these parameters, particularly of ACSL-1, in the liver after treatment with *C. ficifolia extract*.

Although the PPARα role in the metabolism of carbohydrates and lipids is well recognized in the liver, the PPARγ role in this organ, where this factor is expressed at low levels, is less known. *C. ficifolia* extract did not increase liver PPARγ expression in STZ-induced diabetic mice, but in HepG2 cells, this factor of transcription was increased significantly in 50%. PPARγ has been a focus of attention as a transcription factor associated with metabolic syndrome in adipocytes [27]. However, its overexpression in hepatocytes has been associated with hepatosteatosis, regulating several proteins associated with lipid uptake, triglyceride storage, and formation of lipid droplets, such as FABP4, fat-specific protein 27 (FSP27)/Cidec, CD36, monoacylglycerol O-acyltransferase 1, and perilipin 2 ([14]. PPARγ is one of the typical phenotypes of steatotic animals associated with obesity and without obesity, which lack triglyceride-storing capacity in adipocytes [28] After pancreaticoduodenectomy, similar overexpression of hepatic PPARγ was observed in generally non-obese NAFLD/NASH patients [14] [28].

Since activation of PPARγ is steatogenic, the *C. ficifolia* extract null effect on PPARγ in diabetic mice may be considered beneficial. However, treating genetically obese or diet induced NAFLD/NASH mice with PPARγ ligands also can decrease hepatic triglycerides. This distinct effect may be attributed to an enhanced adiponectin synthesis in adipose tissue [14]. Circulating adiponectin could increase glucose uptake and lipid oxidation in hepatocytes by activating AMPK, thereby improving systemic insulin sensitivity, and reducing liver steatosis [14].

However, *C. ficifolia* extract did not significantly affect adiponectin in 3T3-L1 adipocytes.

PPARγ activation reduces inflammatory response by negatively interfering with NF-κB and signal transducers and transcriptional activators [14], suppressing the production of pro-inflammatory cytokines in 20%, including TNF-α. PPARγ also has significant anti-inflammatory properties, which can regulate the inflammatory immune response. *C. ficifolia* extract reduced pro-inflammatory cytokines TNF-α and IL-6 expression in 20% at 25% in STZ-induced diabetic mice.

The *glut*-1 promotor in HepG2 cells has regions of response to the transcription complex HIF-1α/ARNT [29]. In the case of GLUT-1, this transporter might be participating in the *C. ficifolia* extract hypoglycemic effect, promoting glucose intake in hepatocytes and probably in other tissues that also express this transporter, like brain, muscle, pancreas, and adipose tissue, contributing to glycemic control in the organism [30] This effect on GLUT-1 expression due to *C. ficifolia* extract may be associated with an enhancement in HIF-1α/ARNT pathway, better than with PPARs. Therefore, this cellular line may not be suitable for studying PPARα activation, given its carcinogenic origin and hypoxia characteristics. Further studies will be necessary using primary cultures of hepatocytes for to clarify the participation of these pathways in the effects of *C. ficifolia* extract in liver, as well as study the participation of other glucose transporters, as GLUT-2, in the effects of *C. ficifolia* extract.

In 3T3-L1 adipocytes, PPARγ expression was not significantly affected. Adipogenesis in 3T3-L1 cells is preceded by an increase in C/EBP-β and CEBP-Δ expression, whose posterior reduction is associated with an increase in C/EBP-α and PPARγ. PPARγ expression in adipocytes by *C. ficifolia* extract did not change, probably due to the process of adipogenesis at eight days, beginning with PPARγ constant expression levels that promote the adipogenesis and expression of genes, like GLUT-4.

C. ficifolia extract significantly promoted FATP-1 expression, probably associated with the non-significant increase in PPARγ observed in 3T3-L1 adipocytes. All data suggest that *C. ficifolia* extract promotes the activation of PPARs in adipocytes; the increase in FATP-1 (2-fold) and GLUT-4 (1.5-fold) expression in these cells by *C. ficifolia* extract may explain part of the hypoglycemic effect, enhancing glucose and fatty acids, as well as its storage as triglycerides, as it has been observed.

D-chiro-inositol is a glycan, a second messenger in the classic insulin route. Its administration improves glucose tolerance, insulin resistance, and type 2 diabetes [31]. The extract of *C ficifolia* contained 3.32 mg/g of extract, and this quantity was used as a reference to calculate the concentrations used in all the treatments with *C. ficifolia* extract. Given the polar nature of D-chiro inositol, in contrast with the lipophilic nature of the ligands of PPARs, it is possible that other compounds, apart from D-chiro-inositol, also are participating in these effects of *C. ficifolia* extract, like β-sitosterol and 4-hydroxybenzoic acid that were isolated from *C. ficifolia*, exhibiting insulin secretagogue action in RINm5F and

activation of PPARγ in C2C12 myocytes [11], increasing expression of GLUT-4, which may be contributing to the hypoglycemic action of *C. ficifolia extract*. This effect on GLUT-4 also was observed in 3T3-L1 adipocytes by *C. ficifolia extract.*

Also, some terpenoids have been suggested as dual agonists of PPAR-α/PPAR-γ, particularly isoprenyl-like farnesol, and geranylgeraniol, with dual activity in HepG2 and 3T3-L1 [32]. In this context, cucurbitacins of *C. ficifolia*, triterpenes characterized by the presence of a cucurbitacin nucleus (19-(10 → 9β)-abeo-10α-lanostano-5-ene), described in the Cucurbitaceae family, might be participating in these actions [33] [34]. However, the presence of these compounds and their participation in these actions should be studied in posterior studies.

5. Conclusion

In conclusion, the aqueous extract from fruit of *C. ficifolia* contains PPAR agonists that modify the glucose metabolism of carbohydrates and lipids, implicating diverse cell types associated with its metabolism, such as hepatocytes, adipocytes, and skeletal muscle cells, with a capacity to stabling altered processes in pathologies associated with chronic inflammatory response characteristic of obesity, type 2 diabetes, and other metabolic diseases.

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Conflicts of Interest

The authors declare no conflict of interest.

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