



Cucurbita ficifolia regulates the secretomes of adipocytes and macrophages in co-culture, breaking meta-inflammation

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Abstract

Cucurbita ficifolia's fruit aqueous extract has been attributed with antidiabetic and anti-inflammatory properties, offering a promising alternative in addressing meta-inflammation. Meta-inflammation plays a pivotal role in the development of metabolic dysfunction, contributes to conditions such as obesity, diabetes mellitus, and metabolic syndrome. The interactions between adipocytes and macrophages within adipose tissue cause the onset of meta-inflammation, leading to disruptions in the secretomes and transcriptomes of inflammatory cytokines among other factors. We evaluated the effects of the aqueous extract from the fruit of *Cucurbita ficifolia* on the secretomes and transcriptomes of 3T3-L1 adipocytes and RAW-264.7 macrophages, by exchange of their conditioned mediums and in co-culture conditions. Adipocytes incubated with conditionate medium from macrophages treated with the extract exhibited an increase in GLUT-4 expression, and the protein release of TNF α , accompanied by a reduction in IL-1 β . In contrast, macrophages incubated with conditionate medium from adipocytes treated with the extract exhibited inhibition the release of TNF α , IL-6, and IL-10, with TLR-4 expression reduction. Interestingly, under the conditions of indirect simultaneous co-culture, the release of TNF α , IL-6, and IL-1 β was disrupted, increasing PPAR γ expression in 3T3-L1 adipocytes. The salicin, compound reported in *Cucurbita ficifolia* extract, had a similar effect in this last model. The aqueous extract of *Cucurbita ficifolia* demonstrates the ability to suppress meta-inflammation by correcting the secretion patterns of TNF α and IL-6 in co-culture conditions. It effectively modulates the interactions between 3T3-L1 adipocytes and RAW-264.7 macrophages, leading to an improvement in the inflammatory cytokines profile.

Keywords *Cucurbita ficifolia* · Obesity · Inflammatory cytokines · Meta-inflammation · Adipocytes · Macrophages

Introduction

Cucurbita ficifolia Bouché (Cucurbitaceae) is a plant grown for its edible fruits and it is utilized in Mexican traditional medicine as an antidiabetic remedy. Several pharmacological

studies in vivo and in vitro, and in type 2 diabetes patients support its medicinal properties (Acosta-Patiño et al. 2001; Alarcon-Aguilar et al. 2002). In sub-acute in vivo studies, the daily administration of an aqueous extract from the fruit of *C. ficifolia* over 30 days in rodents resulted in reduced glycemia due to the accumulation of liver glycogen and increased insulin secretion (Xia and Wang 2006; Garcia-Gonzalez et al. 2017). This effect was further confirmed in vitro using RINm5F cells (Miranda-Perez et al. 2016). Furthermore, the anti-inflammatory properties of *C. ficifolia* extract were demonstrated in streptozotocin-induced diabetes mice (Roman-Ramos et al. 2012; Diaz et al. 2012), in monosodium glutamate-induced obesity mice and in 3T3-L1 adipocytes cultures (Fortis et al. 2013). These studies suggest that the aqueous extract from the fruit of *C. ficifolia* serves as a modulator of inflammatory cytokines. It was found to enhance the expression of IFN γ and IL-10 while decreasing the expression of TNF α , IL-6, and resistin,

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without changes in adiponectin (AdipoQ) expression (Fortis et al. 2013, 2017).

Xia and Wang (2006) proposed D-chiro-inositol (DCI) as one of the key principles responsible for the hypoglycemic effects of *C. ficifolia* (Xia et al. 2006). However, other compounds have also been identified in the fruit extracts, including β -sitosterol, 4-hydroxybenzoic acid, hydroxyphenyl acetic acid, gallic acid, catechin, p-coumaric acid, cinnamic acid, and salicin (Garcia-Gonzalez et al. 2017). In additional studies, β -sitosterol and 4-hydroxybenzoic acid were found to increase insulin secretion in RINm5F cells and enhance the activation and mRNA expression of PPAR γ , a member of the peroxisome proliferator-activated receptors family, in C2C12 myocytes (Rosiles et al. 2022). Salicin is commonly recognized as anti-inflammatory agent (Kong et al. 2014); however, its effect on adipocytes and macrophages in coculture has not been explored.

In obesity, the secretomes from interactions between adipocytes and macrophages in adipose tissue generate a meta-inflammation that plays a fundamental role in the development of metabolic dysfunctions (Svetoslav et al. 2022). Given the demonstrated anti-inflammatory actions of the aqueous extract from the fruit of *C. ficifolia*, it is essential to conduct studies aimed at determining the extract's effects on the secretomes of adipocytes and macrophages in co-culture. These interactions, particularly in the context of obesity, contribute to the generation of meta-inflammation associated with various metabolic diseases. The aim of the present research was to determine the impact of the aqueous extract from the fruit of *C. ficifolia* on the secretomes and transcriptomes of 3T3-L1 adipocytes and RAW-264.7 macrophages. This involved incubating each cellular type with the exchange of conditioned mediums and employing conditions of indirect simultaneous co-culture. The primary goal was to mitigate the meta-inflammation, generated from the interactions in the transcriptomes and secretomes of inflammatory cytokines, lipid transporters, and PPARs, by the *C. ficifolia* extract treatment.

Materials and methods

Preparation of the aqueous extract from fruit of *C. ficifolia*

Fruits were collected from Acolman Municipality, Estado de México, México. Mesocarp free of exocarp and seeds was dehydrated at ambient temperature in a laminar flow hood. The resulting dried material was ground (400 g) and subjected to maceration in 4 L of water for 72 h. After maceration, the extract was filtered (Whatman 4, Merck-Millipore, MO, USA), freeze-dried, and stored at -70°C until use.

Quantification of the content of salicin in the aqueous extract from fruit of *C. ficifolia*

In previous studies, salicin was one of the majoritarian compounds in the aqueous extract of *C. ficifolia*, a glycosidic alcohol with anti-inflammatory activity (Garcia-Gonzalez et al. 2017; Rosiles et al. 2022). Consequently, salicin was chosen as the standard for its quantification in the extract. A calibration curve using various concentrations of a commercial standard of salicin (31.25, 62.5, 125, 250, and 500 $\mu\text{g}/\text{mL}$) was generated through High-Performance Liquid Chromatography (HPLC). The retention factor of the compound and the area under the curve were determined, establishing a salicin content of 0.88 mg/g of extract ($30.63\ \mu\text{M} = 10\ \mu\text{g}/\text{mL}$).

Cell lines cultures

Cell lines of murine origin, specifically 3T3-L1 fibroblasts (ATCC; CL-173) and RAW 264.7 (ATCC; TIB-71) macrophages, were obtained from ATCC (DC, USA). The 3T3-L1 fibroblasts were cultured in DMEM (Sigma-Aldrich, Steinheim, Germany) supplemented with 2 mM of glutamine and 10% newborn calf serum (Gibco, ThermoFisher Scientific, MA, USA) to proliferation stage. In differentiation stage the newborn calf serum was replaced by fetal bovine serum (Gibco, ThermoFisher Scientific, MA, USA). RAW 264.7 cells were cultured in supplemented DMEM with 10% fetal bovine serum and 1% gentamicin antibiotic. All cell cultures were maintained in a standard incubator at 37°C with a humidified atmosphere, containing 5% CO_2 . 3T3-L1 fibroblasts, at 70–80% confluence underwent differentiation into adipocytes. The cells were incubated in supplemented DMEM with 10% bovine fetal serum, isobutyl-1-methylxantina (IBMX, 0.5 mM), dexametazone (25 μM), and 1 $\mu\text{g}/\text{mL}$ of insulin for 2 days. At third day, the medium was replaced, and fresh medium with insulin (without IBMX and dexametazone) was added for an additional 2 days. From the fourth day onward, the culture was maintained with DMEM containing 10% bovine fetal serum, with medium changes every two days. Adipocytes were utilized after eight days of stimulation. Previously to coculture, both cellular lines were independently cultivated and proliferated.

Cell viability assay

A cells viability test using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich, Steinheim, Germany), was conducted following the Mosmann method (Mosmann 1983). Cells, both 3T3-L1 and RAW 264.7, were seeded at a density of 5000 cells/well and

incubated in DMEM at 5% CO₂ and 37 °C. After 24 h, cells were washed with PBS, and the culture medium was added. Subsequently, the cells were incubated with *C. ficifolia* extract (24 h) at different concentrations (1, 10 and 100 µg/mL). Following exposure to *C. ficifolia* extract, the culture medium was recovered and a MTT solution (0.2 mg/mL) in PBS (pH = 7.5) was added. The cells were then incubated for 3 h, at 5% CO₂ and 37 °C. Afterward, the medium was once again collected, and a HCl solution (0.04 M, 200 µL) in isopropanol was added during 15 min incubation to dissolve the formazan. Absorbance was measured at 570 nm using an ELISA plate reader. The results were normalized as a percentage of the control treatment. Notably, the concentration of 10 µg/mL of *C. ficifolia* extract (equivalent 30.63 µM of salicin) demonstrated a higher percentage of viable cells in both cellular lines (data not shown). Consequently, this concentration was selected for further experiments. In subsequent assays, the cells were cultured with an appropriate support, according to the specific requirements of each experiment.

Temporary course of PPAR γ and GLUT-4 mRNA expression in 3T3-L1 adipocytes treated with *C. ficifolia* extract

To understand the temporal dynamic of the expression of the RNA of PPAR γ and GLUT-4 in 3T3-L1 adipocytes, treated with 10 µg/mL of *C. ficifolia* extract, it was performed a kinetic study. Cells were incubated for different times (0.5, 1, 3, 6, 12, and 24 h) to identify the time points of major expression of PPAR γ and GLUT-4 in adipocytes. The determination of PPAR γ and GLUT-4 expression levels was carried out as explained below. Treatment with *C. ficifolia* extract caused an increase of mRNA expression of PPAR γ , reaching its highest level at 0.5 h of incubation, followed by a gradually decreased until the 24 h. Simultaneously, the *C. ficifolia* extract caused a reduction in GLUT-4 mRNA expression at 0.5 h and further decreasing at 1 and 3 h, and staying on basal levels ($t=0$) until the 24 h of incubation (supplementary data). Consequently, for the determination of transcriptome and secretome in 3T3-L1 adipocytes with the exchange of mediums from RAW 264.7 macrophages treated with *C. ficifolia* extract for 24 h, the optimal incubation time was determined to be 0.5 h.

Temporary course of TNF α release in the RAW 267.4 macrophages treated with LPS and *C. ficifolia* extract

A kinetic study was conducted to observe the temporal dynamics of TNF α release in RAW 267.4 cells stimulated with 1 µg/mL of lipopolysaccharide (LPS) for 24 h. This aimed to identify the time of TNF α secretion peak in

macrophages. Other cells were also treated with 10 µg/mL of *C. ficifolia*, incubating at different times (0.5, 1, 3, 6, and 24 h). TNF α levels were determined using an ELISA assay (Biolegend, CA, USA). Treatment with LPS induced an increase in TNF α release, reaching its highest level at 1 h of incubation. *C. ficifolia* extract reduced TNF α levels from 0.5 h and consistently throughout the 24 h incubation period (supplementary data). Consequently, for the determination of secretome of RAW 264.7 macrophages with interchange of mediums from 3T3-L1 adipocytes treated for 24 h with *C. ficifolia* extract, the optimal incubation time was determined to be 0.5 h.

Cellular cultures with interchange of conditioned mediums

A cell culture model with exchange of conditioned mediums was established to investigate the effect on cellular transcriptomes and secretomes as indicated below. After incubating one of the cellular lines with 10 µg/mL of *C. ficifolia* extract for 24 h, the culture medium of the other cellular line was replaced with the conditioned medium. For the analysis of secretomes and transcriptomes in 3T3-L1 cells and RAW 264.7 cells, the incubation time with the mediums was set at 0.5 h. In these experiments, three groups were studied for each cellular line: Group 1: cells treated with the vehicle (control); Group 2: cells with the interchange of medium (negative control); Group 3: cells with the interchange of conditioned medium (previously treated with *C. ficifolia* extract for 24 h). In the experiments with RAW 264.7 macrophages, the use of LPS was omitted. Instead, these macrophages were stimulated with the culture medium derived from 3T3-L1 adipocytes treated with *C. ficifolia* extract. This adipocyte medium contains fatty acids released by the cells, which act as stimulus for TLR-4 receptors like LPS (Chait and Kim 2010).

Cellular cultures in conditions of indirect simultaneous coculture with treatment of *C. ficifolia* extract and salicin

In conditions of indirect simultaneous coculture, Corning Costar Transwell devices (Corning Costar, NY, USA), with polycarbonate membrane and 0.4 µm pore (CLS3450, Sigma, Steinheim, Germany) were used. This device effectively separate adipocytes from macrophages, facilitating the exchange of secretomes. The 3T3-L1 cellular line was cultured in 6-wells plates, while RAW 264.7 cells were cultivated in the hanging device or insert of the Transwell system. This arrangement allowed for simultaneous exchange of the culture medium. The Transwell system inserts-maintained separation of cell types while facilitating the exchange of soluble molecules. In these experiments, three groups were

studied for each cellular line: Group 1: cells simultaneously incubated for 24 h with the vehicle (control); Group 2: cells incubated for 24 h with *C. ficifolia* extract; Group 3: cells incubated for 24 h with salicin. In all cases, both cultured cells and supernatant fractions were harvested for RT–PCR, and ELISA, respectively.

Secretomes and transcriptomes analysis of 3T3-L1 adipocytes and RAW 264.7

For secretomes, the quantification of TNF- α , IL-6, IL-1 β , and IL-10 levels in the culture mediums was conducted using ELISA kits from Biolegend (Biolegend, CA, USA). The procedures followed the instructions provided by the supplier. For transcriptomes, the mRNA expression of FATP1, GLUT-4, PPAR γ , PPAR α , TLR-4, AdipoQ, TNF- α , IL-6, and IL-10 was performed as follow: RNA was extracted using the TRIZOL method, and it was quantified in NanoDrop 2000 (Thermo Fisher Scientific, WI, USA). Two micrograms of total RNA underwent reverse transcription using the ImProm II reverse transcription system (Promega, Madison, WI, USA). The reaction (2 μ g of total RNA) was incubated in a thermocycler (Thermo Rotor Gen, Corbett Res, CA, 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. Subsequently, cDNAs were amplified using SYBR Green master mix (Roche Molecular Biochemicals, Mannheim Germany) containing 0.5 mM of customizing primers

(Table 1) for FATP-1, GLUT-4, PPAR γ , PPAR α , TLR-4, adiponectin (AdipoQ), TNF- α , IL-6, and IL-10, and as internal control, 36B4, each one plus Fast Star Enzyme, PCR buffer and 3.5 mM MgCl₂ in a final volume of 10 μ L. The reactions were measured in a rotor gene system. PCR was conducted under the following cycling conditions: enzyme pre-incubation during 10 min at 95 °C, 35 or 40 cycles denaturizing at 95 °C for 10 s, a thermal ramp rate of 20 °C per sec; annealing at 61 °C for 7 s with thermal ramp rate of 20 °C per sec; amplification at 71 °C for 10 s with a thermal ramp rate at 20 °C per sec. The threshold cycles (Ct) were measured in separated tubes and in duplicate. To verify the identity and purity of the amplified products, electrophoresis was performed on 2% agarose mini gels. Additionally, and analysis of the melting curve was carried out at the end of amplification under following conditions: denaturation at 95 °C, with a thermal ramp rate of 20 °C per sec, re-annealing at 65 °C for 15 s, 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. To guarantee the quantity of the measurements, each assay included a negative control for each gene. The abundance of the cytokines' mRNA was normalized by the abundance of the encoding 36B4 mRNA. The Δ Ct values were calculated in every sample for each gene of interest as follows: Ct interest—Ct reference with 36B4 as the reference gene (mRNA of reference remained stable throughout the experiments). Relative changes in the expression level of each gene ($\Delta\Delta$ Ct) were calculated as

Table 1 Genes and primers used in 3T3-L1 adipocytes and RAW 264.7 macrophages

Genes	Primer forward	Primer reverse	Pb
TNF α <i>Gene bank access NM_013693.2</i>	ACTTGGTGGTTTGCTACGAC	CCTCCCTGTCATCAGTTCTA	102
IL-6 <i>Gene bank access NM_031168.1</i>	TTCCATCCAGTTGCCTTCTT	CAGAATTGCCATTGCACAAC	129
IL-10 <i>Gene Bank NM_010548.2</i>	GGTGAGAAGCTGAAGACCCCT	TGTCTAGGTCCTGGAGTCCA	248
TLR-4 <i>Gene Bank NM_021297.3</i>	TCTAACTTCCCTCCTGCGAC	ACGATCTGTAAGTGGTGCA	174
PPAR- γ <i>Gene bank access NM_011146.1</i>	CCAGAGTCTGCTGATCTGCG	GCCACCTCTTTGCTCTGCTC	217
PPAR- α <i>Gene bank access NM_011144</i>	ATGCCAGTACTGCCGTTTTC	GGCCTTGACCTTGTTTCATGT	220
GLUT-4 <i>Gene Bank NM_009204.2</i>	GATTCTGCTGCCCTTCTGTC	ATTGGACGCTCTCTCTCCAA	168
FATP-1 <i>Gene Bank NM_011977.3</i>	ACCAGTGTCCAGGGGTACAG	TGTCTCCCAGCTGACATGAG	174
Adiponectin (Adipo Q) <i>Gene Bank NM_009605.4</i>	GGCTCTGTGCTCCTCCATCT	AGAGTCGTTGACGTTATCTGCATAG	101
36B4 <i>Gene Bank NM_007475.2</i>	AAGCGCGTCCTGGCATTGTCT	CCGCAGGGGCAGCAGTGGT	135

ΔCt sample minus ΔCt reference and then presented as $2^{-\Delta\Delta\text{Ct}}$ (Dehoux et al. 2003).

Statistical analysis

Data are presented as the mean $\pm$ S.E.M. Statistical differences among the treatments were determined by an analysis of variance and the Tukey–Kramer Multiple Comparison post-hoc test ($p < 0.05$). All statistical data were computed using the NCSS 2000 software.

Results

Transcriptome analysis of 3T3-L1 adipocytes exposed to conditioned medium from RAW 264.7 macrophages

Figure 1 shows the changes in the transcriptome of 3T3-L1 adipocytes (mRNA expression of PPAR γ , PPAR α , FATP1, GLUT-4, TNF- α , IL-6, and AdipoQ) after the treatment with conditioned medium of RAW 264.7 macrophages incubated with the *C. ficifolia* extract. Exchange of culture mediums from macrophages to adipocytes (negative control) showed a non-significant increase of PPAR γ expression compared with adipocytes without mediums exchange (control). In contrast, treatment of 3T3-L1 cells with conditioned medium of RAW 264.7 macrophages, previously treated with *C. ficifolia* extract caused a significant reduction of PPAR γ (Fig. 1A). In relation to PPAR α , its expression showed a significant reduction in 3T3-L1 adipocytes with medium exchange of macrophages, exhibiting a similar effect than with conditioned medium (Fig. 1B). The transcriptome analysis revealed that the expression of GLUT-4, FATP-1, TNF- α , and IL-6 in control 3T3-L1 adipocytes was significantly lower than in cellular cultures with exchange of mediums ($p < 0.05$). For GLUT-4, although both macrophages' mediums, non-conditioned and conditioned, exhibited increased expression compared with control, the increase observed with the conditioned medium was superior (Fig. 1C). For FATP-1, both mediums significantly increased its expression; however, the increase observed with the conditioned medium was minor (Fig. 1D). In relation to TNF- α and IL-6, the expression levels were significantly increased in the cells with mediums exchange, with a notably superior increase observed in the case of the conditioned medium (Fig. 1E–F). AdipoQ expression didn't have any modification compared with control adipocyte culture (Fig. 1G).

Secretome of 3T3-L1 adipocytes incubated with conditioned medium of RAW 264.7 macrophages

Figure 2 shows the changes in the secretomes of 3T3-L1 adipocytes (concentrations in culture mediums of TNF- α , IL-6, IL-10, and IL-1 β) after of the treatment with conditioned medium of RAW 264.7 macrophages incubated with *C. ficifolia* extract. Release of TNF- α , IL-6, and IL-10 was significantly minor in cells with mediums' exchange, non-conditioned or conditioned, compared with control (Fig. 2A–C); in these cases, the observed reduction was less evident with the conditioned medium. IL-1 β release was significantly increased in adipocytes incubated with the macrophages medium and significantly decreased with the conditioned medium ($p < 0.05$), in both cases compared with control adipocyte cultures (Fig. 2D).

Transcriptome of RAW 267.4 macrophages incubated with conditioned medium of 3T3-L1 adipocytes

Figure 3 shows the changes in the transcriptomes of RAW 267.4 macrophages (mRNA expression of TNF- α , IL-6, IL-10, and TLR4), after of the treatment with conditioned medium of 3T3-L1 adipocytes treated with *C. ficifolia* extract. TNF- α and IL-6 mRNA expressions were significantly increased in RAW 264.7 macrophages only when incubated with conditioned medium compared with control (Fig. 3A–B). In contrast, IL-10 significantly increased with the adipocyte medium and decreased in conditioned medium compared with control (Fig. 3C). TLR4 mRNA expression was significantly decreased in RAW 264.7 macrophages incubated in conditioned medium of 3T3-L1 cells treated with *C. ficifolia* (Fig. 3D), compared with negative control.

Secretome of RAW 264.7 macrophages incubated with conditioned medium of 3T3-L1 adipocytes

Figure 4 shows the changes in the secretomes of RAW 264.7 macrophages (TNF- α , IL-6, IL-10), incubated with conditioned medium of 3T3-L1 adipocytes treated with *C. ficifolia* extract. In the adipocytes medium, TNF- α and IL-6 release stayed without changes, whereas IL-10 was significantly increased compared with control (Fig. 4A–C). In conditioned medium, TNF- α , IL-6, and IL-10 release were significantly decreased in RAW 267.4 macrophages compared with control and negative control (Fig. 4A–C).

Fig. 1 Transcriptome changes in 3T3-L1 adipocytes (mRNA relative expression of PPAR γ , PPAR α , GLUT-4, FATP1, TNF- α , IL-6, and adiponectin (AdipoQ)) after of the treatment with conditioned medium of RAW 264.7 macrophages incubated with the *C. ficifolia* extract. Control: 3T3-L1 adipocyte culture; Negative control: 3T3-L1 adipocyte culture treated with medium of RAW 264.7 macrophages; *C. ficifolia*: 3T3-L1 adipocyte culture treated with conditioned medium of RAW 264.7 macrophages incubated with *C. ficifolia* extract (Mean $\pm$ S.E., $n=4$). *Significant difference against control ($p<0.05$); &Significant difference against negative control ($p<0.05$)

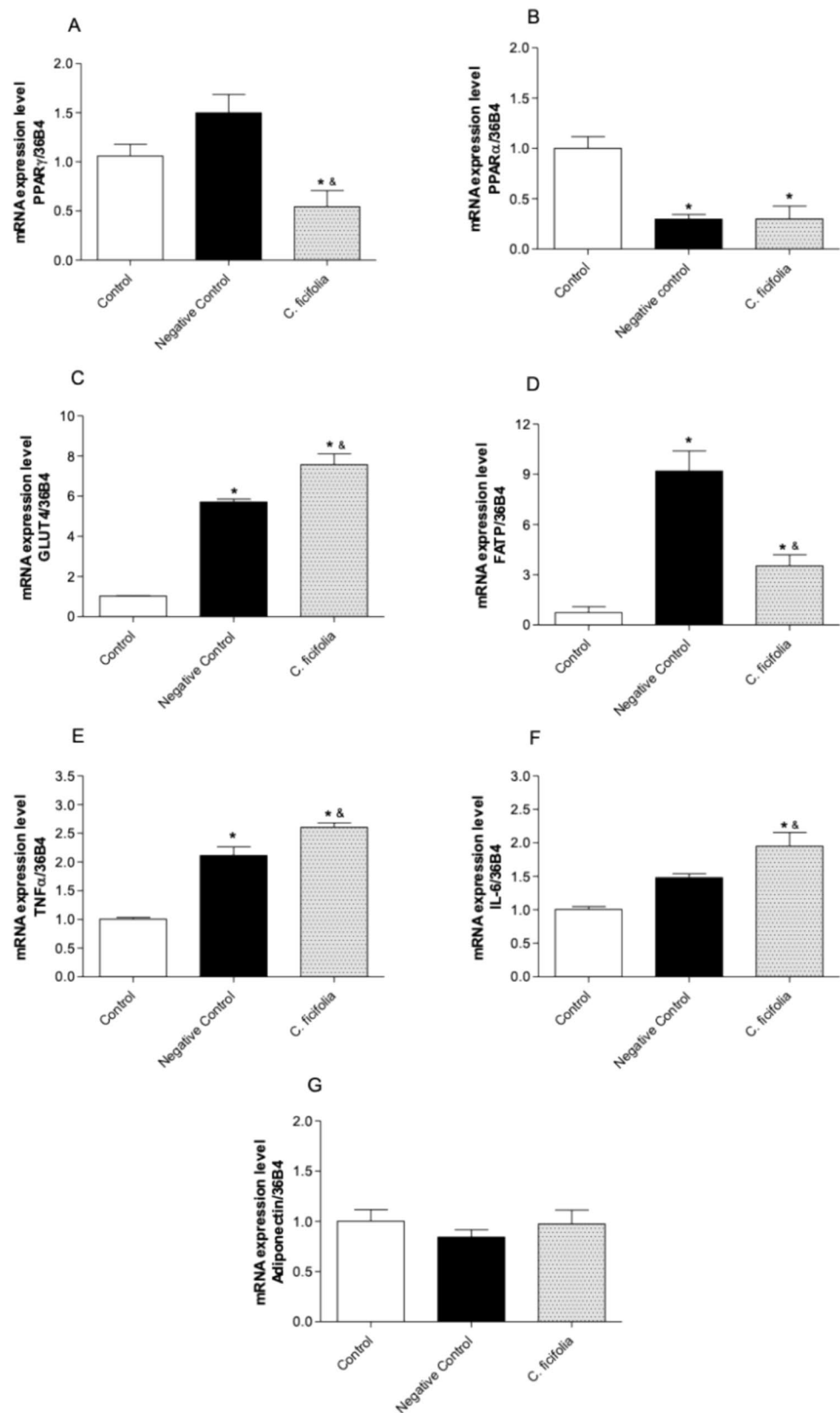


Fig. 2 Secretome changes in 3T3-L1 adipocytes (TNF- α , IL-6, IL-10, and IL-1 β) after of the treatment with conditioned medium of RAW 264.7 macrophages incubated with the *C. ficifolia* extract. Control: adipocyte culture; Negative control: adipocyte culture treated with medium of RAW 264.7 macrophages; *C. ficifolia*: adipocyte culture treated with conditioned medium of RAW 264.7 macrophages incubated with *C. ficifolia* extract. (Mean $\pm$ S.E., $n=4$). *Significant difference against control ($p<0.05$); &Significant difference against negative control ($p<0.05$)

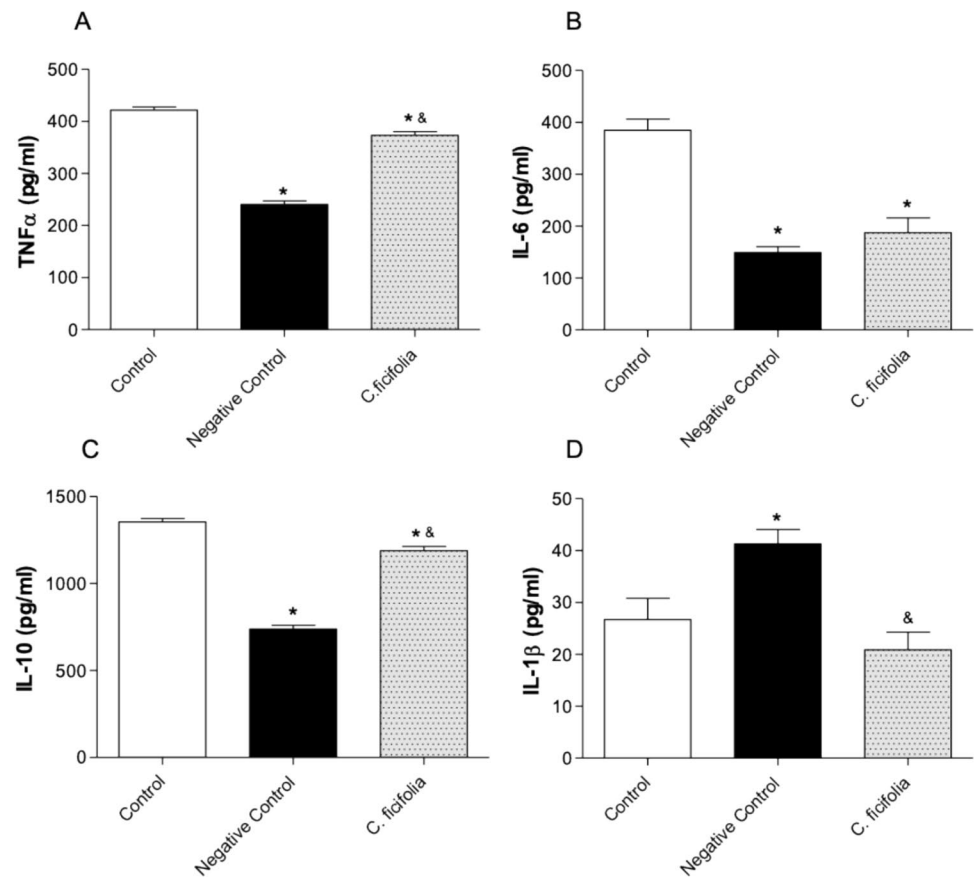
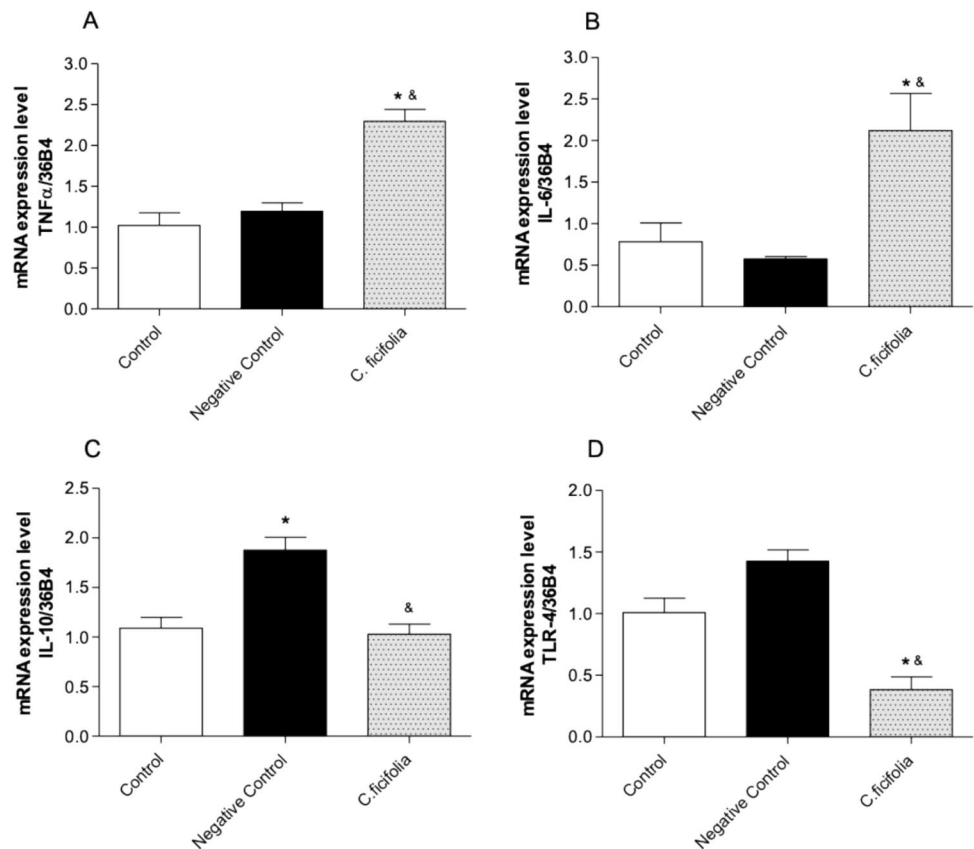


Fig. 3 Transcriptome changes in RAW 264.7 macrophages (mRNA relative expression TNF- α , IL-6, IL-10, and TLR-4) after of the treatment with conditioned medium of 3T3-L1 adipocytes incubated with the *C. ficifolia* extract. Control: RAW 264.7 macrophage culture; Negative control: RAW 264.7 macrophage culture treated with medium of 3T3-L1 adipocytes; *C. ficifolia*: RAW 264.7 macrophage culture treated with conditioned medium of 3T3-L1 adipocytes incubated with *C. ficifolia* extract. (Mean $\pm$ S.E., $n=4$); *Significant difference against control ($p<0.05$); &Significant difference against negative control ($p<0.05$)



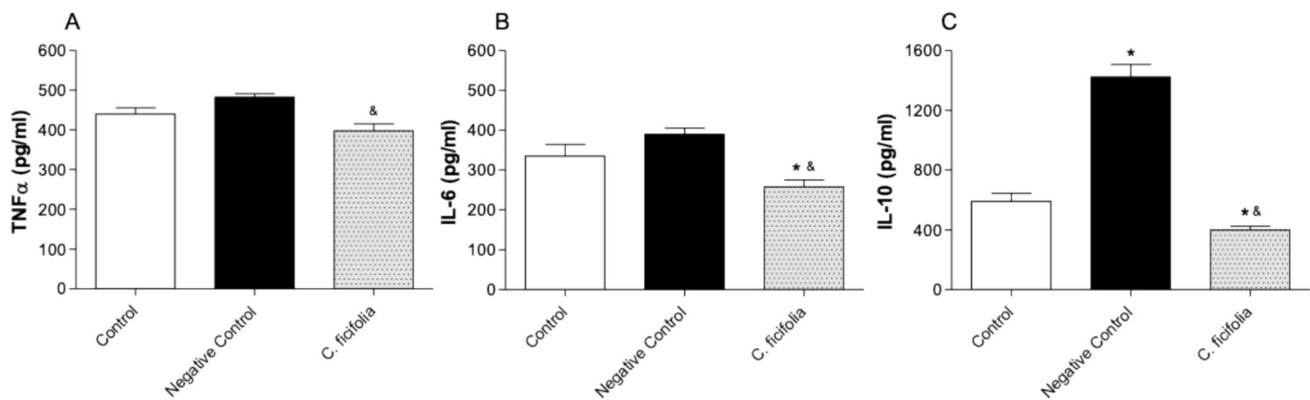
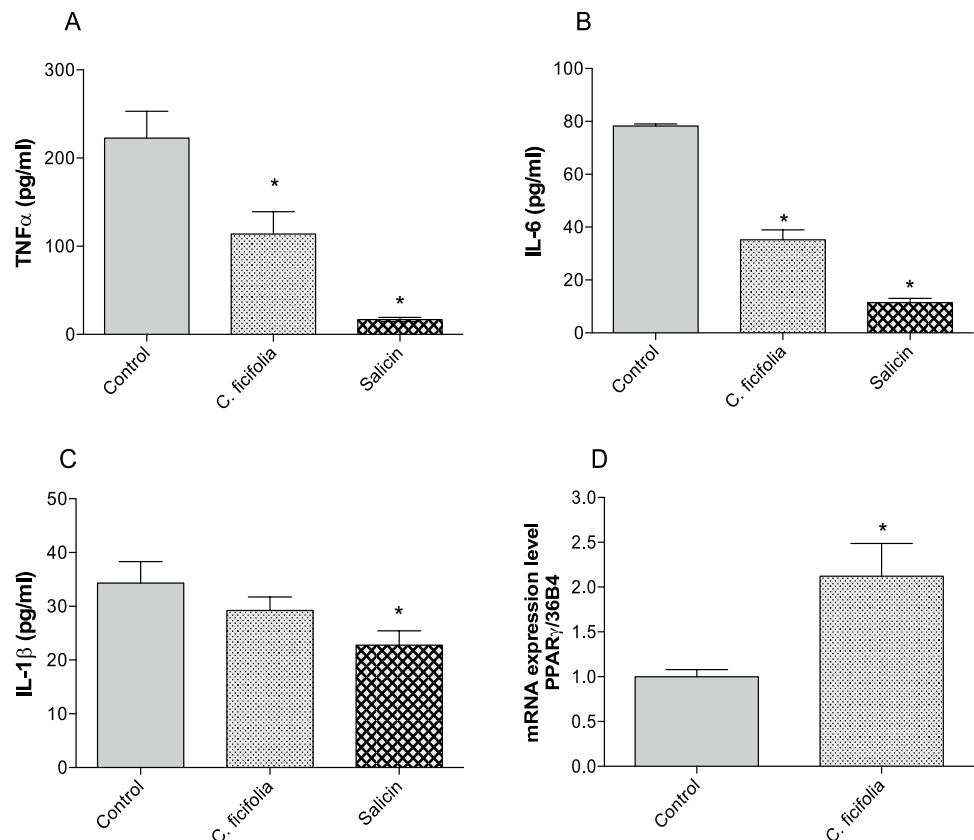


Fig. 4 Secretome changes in RAW 264.7 macrophages (TNF- α , IL-6, IL-10) after the treatment with conditioned medium of 3T3-L1 adipocytes incubated with the *C. ficifolia* extract. Control: RAW 264.7 macrophage culture; Negative control: RAW 264.7 macrophage culture treated with medium of 3T3-L1 adipocytes; *C. ficifolia*: RAW

264.7 macrophage culture treated with conditioned medium of 3T3-L1 adipocytes incubated with *C. ficifolia* extract. (Mean $\pm$ S.E., $n=4$). *Significant difference against control ($p<0.05$); &Significant difference against negative control ($p<0.05$)

Fig. 5 Secretome changes (TNF- α , IL-6, and IL-1 β) of 3T3-L1 adipocytes and RAW 264.7 macrophages in conditions of indirect simultaneous co-culture, incubated with *C. ficifolia* extract and salicin. Control: 3T3-L1 adipocytes and RAW 264.7 macrophages in conditions of indirect simultaneous co-culture; *C. ficifolia*: 3T3-L1 adipocytes and RAW 264.7 macrophages in conditions of indirect simultaneous co-culture, incubated with *C. ficifolia* extract; Salicin: 3T3-L1 adipocytes and RAW 264.7 macrophages in conditions of indirect simultaneous co-culture, incubated with salicin. (Mean $\pm$ S.E., $n=4$). *Significant difference against control ($p<0.05$)



Secretome and PPAR γ of 3T3-L1 adipocytes and RAW 264.7 macrophages in simultaneous coculture incubated with *C. ficifolia* and salicin

Figure 5 shows the changes in the secretome (TNF- α , IL-6, and IL-1 β) of 3T3-L1 adipocytes and RAW 264.7

macrophages in conditions of indirect simultaneous co-culture, incubated with *C. ficifolia* extract and salicin. TNF- α and IL-6 levels were significantly reduced ($P<0.05$) with both treatments compared with control (Fig. 5A–B). *C. ficifolia* extract caused a non-significant reduction of IL-1 β levels compared with control, whereas

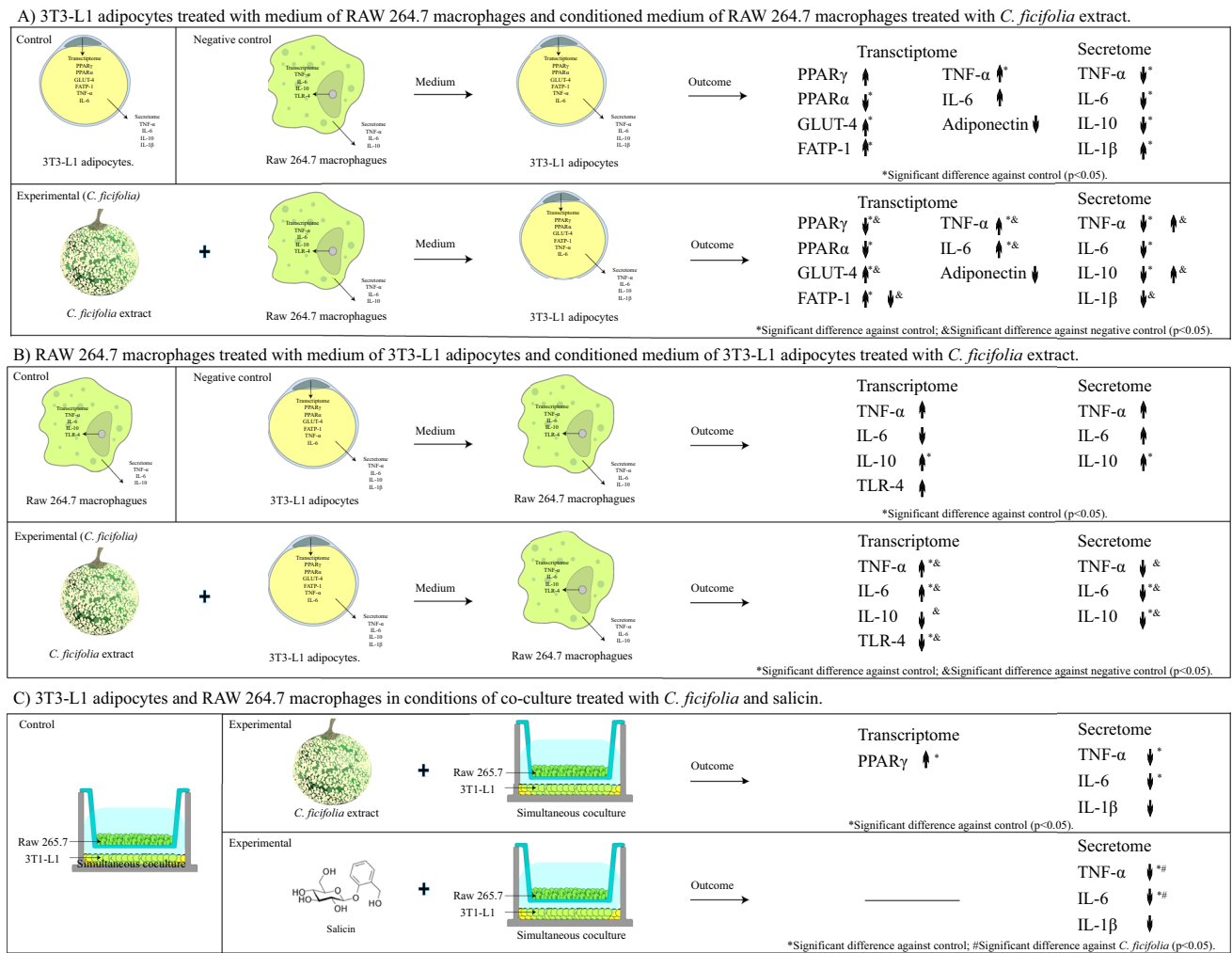


Fig. 6 Summary of the obtained data on transcriptomes and secretomes in adipocytes 3T3-L1 and RAW 264.7 macrophages under the different experimental situations tested of exchange of mediums and in conditions of co-culture. **A** 3T3-L1 adipocytes treated with medium of RAW 264.7 macrophages and conditioned medium of RAW 264.7 macrophages treated with *C. ficifolia* extract. **B** RAW 264.7 macrophages treated with medium of 3T3-L1 adipocytes and conditioned medium of 3T3-L1 adipocytes treated with *C. ficifolia* extract. **C** 3T3-L1 adipocytes and RAW 264.7 macrophages in conditions of co-culture treated with *C. ficifolia* and salicin. *Significant difference against control ($p < 0.05$). &Significant difference against negative control ($p < 0.05$). #Significant difference against *C. ficifolia* ($p < 0.05$)

salicin significantly reduced this pro-inflammatory parameter (Fig. 5C). *C. ficifolia* extract also statistically increased the mRNA expression of PPAR γ (Fig. 5D). Figure 6 summarizes the obtained data on transcriptomes and secretomes in adipocytes 3T3-L1 and RAW 264.7 macrophages under the different experimental situations tested of exchange of mediums and in conditions of co-culture.

Discussion

Obesity is characterized by a chronic subclinical inflammation, commonly referred to as meta-inflammation believed to originate in adipose tissue and intricately connected to the progression of obesity-related comorbidities (Gomes et al. 2021). Obesity associates with macrophage infiltration in adipose tissue. Within this context, infiltrating macrophages interact with adipocytes, collectively contributing to a systemic state of chronic metabolic inflammation, in which the adipocytes and macrophages produce, secrete, and respond to their own stimuli, each cellular type interacts with each other, affecting the lipid and carbohydrate metabolism (Gomes et al. 2021; Xie

et al. 2020). The proximity of adipocytes and macrophages in obese individuals' adipose tissue, triggered the interest for the bidirectional interaction between these cells (Gomes et al. 2021), since regulation of these interactions and their affected transcriptomes and secretomes, may be considered as a therapeutic alternative in the control of metabolic disease.

We have demonstrated in previous studies that the aqueous extract from fruit of *C. ficifolia* induces hypoglycemic, hypolipidemic, antioxidant, and anti-inflammatory effects in different in vivo and in vitro experimental models, including 3T3-L1 adipocytes and C2C12 myocytes (Roman-Ramos et al. 2012; Diaz et al. 2012; Fortis et al. 2013; Rosiles et al. 2022). The present study aims to investigate the effects of *C. ficifolia* on the transcriptomes and secretomes involved in the crosstalk between adipocytes and macrophages, using conditioned mediums exchange and indirect simultaneous co-cultures.

We hypothesized that *C. ficifolia* could regulate the communication between macrophages and adipocytes, thereby influencing cytokine production and release, glucose, and lipid transporters, as well as the expression of PPARs.

To test this hypothesis, we employed a conditioned mediums exchange model. In this model, 3T3-L1 adipocytes were exposed to the secretome from RAW 264.7 macrophages, both with and without treatment of *C. ficifolia* extract. The objective was to study the effects on the transcriptome and secretome associated with inflammatory factors in adipocytes, including PPAR γ , PPAR α , GLUT-4, FATP-1, TNF- α , IL-6 and AdipoQ. In other model, the cells were reverted, where RAW 264.7 macrophages were exposed to secretome from 3T3-L1 adipocytes with and without treatment of *C. ficifolia* extract, also studying the transcriptome and secretome associated with inflammatory factors of macrophages, like TNF- α , IL-6, IL-10, IL-1 β , and TLR-4. Finally, we investigated an indirect simultaneous co-culture model involving both cell types after treatment with *C. ficifolia* extract and salicin, one of the main components in the extract. In general, extract decreased the secretomes of RAW 264.7 macrophages and 3T3-L1 adipocytes in conditions of exchange of conditioned mediums and simultaneous co-culture, causing significant modifications in TNF- α , IL-6, IL-1 β , and PPAR γ .

The differential impact of *C. ficifolia* on RAW 264.7 macrophages and 3T3-L1 adipocytes in these experimental conditions was consistent with previous observations that *C. ficifolia* plays a role in the cytokine secretion (Fortis et al. 2013). The conditioned medium from RAW 264.7 macrophages (negative control) did not impact the PPAR γ , IL-6 nor AdipoQ transcriptome of adipocytes. However, it significantly decreased PPAR α while increasing GLUT-4, FATP-1, and TNF- α ($p < 0.05$). In conjunction, these results suggest a pro-inflammatory profile induced by macrophages on

adipocytes (Fig. 6). Interestingly, the conditioned medium from macrophages on adipocytes reduced the secretome, particularly for TNF- α , IL-6, and IL-10, while increasing IL-1 β . Considering the decrease in IL-10 and the increase in IL-1 β , these data suggest a certain promotion of the inflammation also. However, the relative transcript concentrations in adipocytes remained unchanged when treated with the conditioned medium from macrophages incubated with *C. ficifolia* extract, except for PPAR γ that was significantly decreased and for IL-6 that was decreased against negative control (Fig. 6). Under this condition, the secretome was decreased, particularly for IL-1 β whereas the observed reductions of TNF- α and IL-10 was lesser than in negative control, improving the inflammatory profile.

In contrast, medium of adipocytes in macrophages, significantly increased only IL-10 transcript and secretome levels, without inducing significant changes in TNF- α and IL-6 against control. The conditioned medium of adipocytes (treated with *C. ficifolia* extract), significantly altered the transcriptome against negative control, increasing TNF- α and IL-6, whereas reducing IL-10 and TLR-4. Interestingly, the secretome was significantly reduced by *C. ficifolia* treatment, decreasing concentrations of TNF- α , IL-6, and IL-10 against negative control. The differences found among the transcriptome and secretome for TNF- α and IL-6, might be explained by the time of incubation. In previous studies, the *C. ficifolia* anti-inflammatory activity in adipocytes was reported at 24 and 48 h of (Fortis et al. 2013). Finally, the indirect simultaneous co-culture of adipocytes and macrophages with treatment of *C. ficifolia* extract or salicin resulted in reductions in the concentrations of proinflammatory cytokines like TNF- α , IL-6, and IL-1 β , accompanied by a significant increase in PPAR γ expression (Fig. 6).

Macrophage secretome (negative control) did not significantly alter the expression of PPAR γ or AdipoQ in adipocytes. This is consistent with observations from previous studies conducted on the liver of STZ-induced diabetic mice and 3T3-L1 adipocytes after exposure to *C. ficifolia* extract (Fortis et al. 2017; Almanza et al. 2023). Adipocytes treated with the macrophages secretome exhibited reduced expression of PPAR α compared to control adipocytes. Concurrently, the expression of GLUT-4, FATP-1, TNF- α , and IL-6 was significantly increased. Remarkably, treatment with conditioned medium of macrophages treated with *C. ficifolia* extract reduced both PPARs and FATP-1, increasing GLUT-4, TNF- α , and IL-6 in adipocytes. Therefore, the effect of the secretome of macrophages in the transcriptome of adipocytes suggests deregulation of the inflammatory process and stimulation of the inflammation by *C. ficifolia* extract. Interestingly, although increased inflammation was observed, GLUT-4 also increased, probably as a compensatory response to insulin resistance promoted by pro-inflammatory cytokines. However, this pro-inflammatory

response also depends on incubation times, which should be investigated in further experiments.

PPAR γ expression only increased significantly in conditions of simultaneous coculture, whereas GLUT-4 expression was increased in adipocytes treated with conditioned medium from *C. ficifolia* extract treated macrophages. Despite that the conditioned medium of macrophages treated with *C. ficifolia* did not increase PPAR γ expression in adipocytes, GLUT-4, increased significantly, both in adipocytes incubated with both mediums of macrophages, with or without treatment of *C. ficifolia* extract. The existence of an intermediary produced by macrophages is a possibility, which induce an increment in the expression of this glucose transporter. Compared to control adipocytes, FATP-1 expression significantly increased in adipocytes treated with the medium from macrophages (negative control) and conditioned medium; however, in comparison to the negative control, its expression was significantly reduced with treatment of *C. ficifolia* extract. Therefore, *C. ficifolia* extract alters the expression of the genes that codify for transporters associated with the metabolism of carbohydrates and lipids. Concerning the secretome of adipocytes treated with conditioned medium of macrophages free of *C. ficifolia* extract, TNF- α , IL6, and IL-10 were significantly decreased, whereas IL-1 β was increased compared with control adipocytes. Conditioned medium of macrophages treated with *C. ficifolia* reversed the effects on TNF- α , IL-10, and IL-1 β compared with negative control, whereas that the reduction of the IL-6 concentration was yet more prominent.

To further explore and deepen the study of the bidirectional crosstalk between adipocytes and macrophages, we reversed our experimental model. Conditioned medium from 3T3-L1 adipocytes with and without treatment of *C. ficifolia*, was used to explore whether these secretomes might alter the transcriptomes and secretomes of macrophages. It is well known that LPS activated macrophages release higher levels of pro-inflammatory cytokines and lower levels of anti-inflammatory adipokines/cytokines (Oliver et al. 2009). In our study, LPS was substituted by the conditioned medium of adipocytes, treated or not, with *C. ficifolia*. The culture medium of adipocytes contains fatty acids that stimulate TLR-4 expression, similarly to LPS.

We investigated the impact of conditioned mediums from 3T3-L1 adipocytes on the expression of TNF- α , IL6, IL-10, and TLR-4 and on the secreted factors in the medium of TNF- α , IL6, IL-10, from macrophages. Only the expression of IL-10 in RAW 264.7 macrophages was significantly increased with conditioned medium of adipocytes free of *C. ficifolia* extract treatment. In contrast, transcriptome of macrophages incubated with adipocytes' medium, the conditioned medium of adipocytes treated with *C. ficifolia* extract significantly increased TNF- α and IL6, whereas reduced IL-10 and TLR-4 expression. A reduction of inflammatory

cytokines might be expected in the transcriptome of macrophages by the conditioned medium of adipocytes, due *C. ficifolia* extract has anti-inflammatory effects, without changes in macrophages. Nevertheless, in previous studies, the incubation times for the anti-inflammatory effect of *C. ficifolia* extract in adipocytes was of 24 h, whereas for macrophages with conditioned medium was only of 0.5 h.

Regarding the macrophages' secretome treated with medium of adipocytes, TNF- α , IL6, and IL-10 were increased compared with control adipocytes, but significantly decreased in conditioned medium of adipocytes treated with *C. ficifolia* extract. Therefore, the secretome certainly exhibited the anti-inflammatory effect of the conditioned medium, by incubation with *C. ficifolia*, on the macrophages. This effect let us see that the extract of *C. ficifolia* doesn't affect the transcription level, rather it may be involved in the inactivation or inhibition of the liberation of pro-inflammatory cytokines. Finally, under conditions of simultaneous coculture, both treatments with *C. ficifolia* extract and salicin resulted in a significant reduction in the concentration of proinflammatory cytokines (TNF- α , IL-6, and IL-1 β), which are implicated in the stimulation of the inflammatory process. Also, *C. ficifolia* extract significantly increased PPAR γ expression.

Previous in vivo and in vitro studies have reported the effects of *C. ficifolia* extract on inflammatory mediators in the secretomes of adipocytes (Fortis et al. 2013, 2017). In these studies, *C. ficifolia* was proposed as a cytokine's inflammatory modulator in 3T3-L1 adipocytes, 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 AdipoQ's expression (Fortis et al. 2013, 2017). Also, a recent report demonstrated the regulation of PPAR α in the liver, both in vivo in liver of STZ-induced diabetic mice and in vitro in HepG2 hepatocytes (Almanza et al. 2023). Although IFN γ was not considered in the present study, the reported regulation of *C. ficifolia* on IFN γ might indicate an increase in immune system response to counteract the inflammatory environment. Several lines of evidence support a role for IFN- γ in mediating metabolic complications associated with obesity; IFN- γ promotes lipolysis in cultured mouse adipocytes and inhibits adipocytes differentiation (Gomes et al. 2021). While we cannot exclude the role of other pro and anti-inflammatory mediators, these results suggest that non-inflammatory signaling pathways may be involved in the regulation of meta-inflammation by *C. ficifolia*.

The evidence demonstrates a relationship between *C. ficifolia*, and cellular receptors involved in metabolism, improving metabolic and inflammatory patterns. This reinforces the role of *C. ficifolia* in the immune and metabolic crosstalk within adipose tissue, leading to a reduction in

the expression and secretion of pro-inflammatory cytokines. However, our study has some limitations. First, the same parameters were not measured in all the experimental conditions and second, the activity of the implicated proteins was not confirmed. Both conditions must be confirmed in future studies as we only measured gene expression and concentrations in the medium. In addition, the evaluation of the different molecules found in *C. ficifolia* such as 4-hydroxy benzoic acid, β -sitosterol, p-coumaric acid, gallic acid, D-chiro inositol, etc. and their mixes will be a priority study. In agreement with Gomes et al., (2021), agents in *C. ficifolia*, like salicin and β -sitosterol, could represent attractive candidates for this metabolic immunomodulation in inflammatory obesity (Gomes et al. 2021).

Our data support the hypothesis that *C. ficifolia* regulates communication between macrophages and adipocytes, reducing the secretion of proinflammatory cytokines and possibly regulating the insulin resistance. This is a unique study, because it addresses how *C. ficifolia*, and possibly salicin, progressively affect both macrophage and adipocyte function. This is particularly relevant, as this situation likely occurs during the development of obesity in more than 1 billion adults worldwide (Xie et al. 2020). For example, the depression of GLUT4 transcript levels in 3T3-L1 adipocytes by macrophages and macrophage cytokines, along with the observations that macrophage IL-1 β and IL-6 transcript levels were altered by coculture with adipocytes treated with *C. ficifolia* extract (Fig. 6), reaffirm the regulatory impact of this extract on the interaction between these two cell types. Our data suggest that TNF- α , IL-6, IL-1 β may be the more potent contributor under the regulation of *C. ficifolia*. IL-6 inhibits Akt phosphorylation and all these proinflammatory cytokines may impair the adipocyte function and would contribute to the development of insulin resistance, in where IL-1 β and TNF- α act in synergy with IL-6 (Xie et al. 2020).

Conclusions

Adipocytes increased in macrophage the production of pro-inflammatory cytokines. On the other hand, macrophages inhibited the production of proinflammatory cytokines in adipocytes, while increasing GLUT4, without changes in AdipoQ. *C. ficifolia* represents an interesting alternative to regulate alterations due to adipocyte-macrophage interaction in adipose tissue, due to their multifactorial impact on numerous cytokines. Our results contribute to the validation of the metabolic effects of *C. ficifolia* that have been demonstrated at different levels of biological organisation, both in vivo and in vitro studies, and in different cellular types, especially in macrophages and adipocytes from adipose tissue. A thorough exploration of the effects of *C.*

ficifolia on interactions in adipose tissue in obesity can provide potential pharmacologic control points to prevent and treat obesity-related metabolic disorders, especially the epigenetic and transcriptional regulation of the physiological changes resulting from the interplay between macrophages and adipocytes (Li et al. 2020). Two models of indirect and direct coculture of macrophages and adipocytes showed coregulation of the expression of *C. ficifolia* through of their secretomes. Our findings offer novel opportunities to identify new therapeutic targets for adipose tissue that could reverse its dysfunction in obesity.

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Availability of data and materials Raw data were generated and will be provided from the corresponding author on reasonable request.

Declarations

Ethical statements The research was carried out after ethical approval from Comité de Ética Divisional. Universidad Autónoma Metropolitana – Iztapalapa.

Competing interests The authors declare that they have no competing interests.

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