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# Low-toxicity aqueous and ethanolic extracts of *Stevia rebaudiana* Bertoni increase glucose uptake and modulate lipid metabolism and inflammation in key metabolic cell models of obesity and type 2 diabetes

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ABSTRACT

Obesity is a chronic disease that leads to an increased risk for insulin resistance and diabetes, increasing the risk of mortality and morbidity. Natural sweeteners are an alternative in energy management reducing calorie intake. In this work, we have evaluated the bioactivity of extracts of *Stevia rebaudiana* Bertoni grown in the department of Antioquia, Colombia. Adipocytes, hepatocytes, myocytes, macrophages, and pancreatic cells, key cellular models involved in development of obesity and diabetes, were used to show that ethanolic and aqueous extracts of this plant have the advantage of low toxicity and reduce triglyceride content in hepatocytes and adipocytes, modulating the expression of genes involved in lipogenesis and adipogenesis. These extracts also increase glucose uptake in both insulin resistant and non-resistant muscle cells and present anti-inflammatory properties in the macrophage model. The range of biological activities demonstrated *in vitro* indicates that Stevia, beyond its natural sweetness, may improve metabolic disorders associated with obesity and T2DM.

## 1. Introduction

The 2025 International Diabetes Federation (IDF) report shows that 590 million people worldwide have diabetes, and IDF projections show that approximately 853 million will be living with diabetes by 2050. Over 90% of people with diabetes have type 2 diabetes mellitus (T2DM) (IDF Diabetes Atlas, 2025). Excessive weight or obesity are the most important significant risk factors in the development and progression of T2DM in all age groups. Abnormal fat accumulation in the body leads to a cellular and metabolic disorder that contributes to the establishment of a low-grade inflammation that may play a causative role in generating insulin resistance, defective insulin production and alteration of other aspects of energy homeostasis in T2DM development (Chandrasekaran & Weiskirchen, 2024).

Reducing body weight is the most efficient strategy for mitigating the complications and comorbidities of T2DM, however an important contributor to obesity and diabetes is the excessive consumption of sugar-rich foods and world population overconsume added sugars. Sweeteners are an alternative offering advantages in energy management as most of them contain minimal or no energy compared to sugar. Substitution of sugar with sweeteners in food and beverages may be recommended to reduce energy intake and this has led to an increase in the consumption of foods and beverages containing non-caloric sweeteners over the last decades (Cheng et al., 2025). These sweeteners can generally be categorized into natural and artificial substances, when they are extracted directly from the plants or manufactured in the laboratory, respectively.

Artificial sweeteners (AS) are a heterogeneous class of compounds with diverse chemical structures such as aspartame, sucralose, saccharin and sodium cyclamate. The effects of AS on metabolic disorders remain controversial, and an increasing number of studies suggest that AS may have different impacts on body health. The long-term intake of these molecules may affect the balance of gut microbiota, inducing inflammatory responses, impairing liver function, and leading to dysfunction

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of glucose and lipid metabolism. AS also may increase the risk of cancer and alter reproductive system function (Liu et al., 2024; Wang & Lin, 2025; Wu et al., 2025).

Natural sweeteners (NS) include agave nectar, hoodia and Luo Han Guo Monk fruit extracts, and stevia, made from extracts of the plant *S. rebaudiana* (Castro-Muñoz et al., 2022; Kossiva et al., 2024).

Stevia is a natural extract typically obtained from fresh or dried leaves of *S. rebaudiana* Bertoni. Unlike AS, natural sweeteners like Stevia contain different bioactive compounds that are responsible for sweetness and other several beneficial activities. For centuries, Stevia has been used by the native peoples of South America for both sweetening and medicinal purposes (Justina Ray et al.,2020). Stevia leaves contain a group of eight steviol glycosides which are rebaudioside A-E, dulcoside A, stevioside and steviolbioside. These glycosides boast sweetness approximately 200–300 times that of conventional sucrose. The rich phytochemical profile of Stevia also includes biologically active substances such as terpenes, flavonoids, tannins, and polyphenols that contribute to its therapeutic properties. In particular, studies have shown that Stevia exhibits anti-oxidant, anti-inflammatory, anti-hypertensive, anti-tumor, anti-hyperglycemic, anti-obesity, anti-hyperlipidemic, and anti-diarrheal effects (Dharani et al., 2024; Faruqi et al., 2024; Peteliuk et al.,2021; Sadhu et al., 2024).

Although Stevia-derived products are already commercially available and commonly used by the population, the biochemical composition of the plant depends on the geographical region of the growth, and the different mechanisms of action of Stevia extracts are not fully understood (Khiraoui et al., 2017). The objective of this work is to evaluate the bioactivity of ethanolic and aqueous extracts of *S. rebaudiana* grown in the department of Antioquia, Colombia on cellular models relevant to obesity and type 2 diabetes – specifically adipocytes, hepatocytes, myocytes, macrophages and pancreatic cells - to comprehensively analyze potential therapeutic targets that will help us understand the toxicity, effects on triglyceride accumulation, anti-inflammatory, anti--oxidant, hypoglycemic, and insulin secretion stimulation activity and overall potential to improve metabolic disorders associated with obesity and T2DM.

## 2. Materials and methods

### 2.1. Plant material

Dried leaves of *S. rebaudiana* from crops grown in Olaya, Antioquia (Colombia) were used to prepare two crude extracts (aqueous (AE) and ethanolic (EE)) for cell-based assays. For traceability in figures and results, AE and EE were coded as OBE217 and OBE218, respectively.

**Preparation and chemical analysis of the extracts**

### 2.2. Aqueous extract (AE)

Dried leaves were milled and suspended in water at a 1:10 (w/v) ratio, kept at 40 °C under constant agitation for 4 h, filtered, and the filtrate was lyophilized to obtain the crude aqueous extract (dark, pasty solid).

### 2.3. Ethanolic extract (EE)

Milled dried leaves were packed in a cellulose thimble/paper filter and extracted in a Soxhlet apparatus with ethanol for 12 h (counted from the onset of distillation). The eluate was concentrated under reduced pressure at 50 °C, and residual solvent/water was removed by further drying at 65 °C to yield a dark, hard solid.

### 2.4. Chemical characterization of Extracts

Steviol glycosides were quantified by HPLC-UV (DAD, 210 nm) (AOXLAB S.A.S.; internal method code PROC-TC−083). Quantitation is expressed as g/100 g extract.

### 2.5. Cell cultures

J774A.1 (TIB−67™), C2C12 (ATCCCRL−1772), 3T3-L1 (CL−173™), Beta-TC−6 (CRL−3605 ™), and HepG2 (HB−8065 ™), were purchased from ATCC (Manassas, VA, USA). Cells were cultured in DMEM with 10% fetal bovine serum (FBS), 2 mM glutamine and 1% penicillin/streptomycin (Sigma- Aldrich, St. Louis, Mo, USA) (Growth Medium – GM) at 37°C and 5% $CO_2$. J774A.1 and HepG2 cells were cultured in GM with 5.5 mM glucose (Sigma- Aldrich, St. Louis, Mo, USA) (GM1); 3T3-L1 and C2C12 cells were cultured in GM with 25 mM glucose (GM2). Beta-TC−6 cells were cultured in GM with 500 μM sodium pyruvate (Sigma- Aldrich, St. Louis, Mo, USA), and 25 mM glucose (GM3).

### 2.6. Cell viability

Adipocytes, myotubes, macrophages, hepatocytes and Beta-TC−6 cells were treated at different concentrations of the Stevia extracts (OBE217 and OBE218) according to the cell type (12.5, 25, 50, 100, 200, 400, and 800 μg/ml) and a MTT Cell Viability Assay Kit (Sigma-Aldrich, St. Louis, Mo, USA) was used. MTT was added to the cells for 2 h. After that time, formazan crystals were dissolved by adding DMSO (Sigma-Aldrich, St. Louis, Mo, USA). Absorbance was measured at 570 nm in a Varioskan ™ LUX microplate multilector (Thermo Fisher Scientific, Waltham, MA, USA). Results from this test established the concentrations of Stevia extracts used to analyze their biological effects.

### 2.7. Macrophage cell culture and activation

J774A.1 cells were cultured in GM1. At 80% confluence J774A.1 cells were incubated in GM1 containing 100 ng/ml LPS (Sigma- Aldrich, St. Louis, Mo, USA) and 20 ng/ml IFN-γ (Sigma- Aldrich, St. Louis, Mo, USA) (Activation medium - AM) for 24 h. Treatments were added during the last 6 h. Dihydrorhodamine (DHR) assay were performed, supernatants were collected to evaluate cytokine concentration, and RNA was collected to evaluate inflammation related gene transcription.

### 2.8. Adipocyte cell culture and differentiation

3T3-L1 pre-adipocytes were cultured in GM2 and differentiated at 2 days' post-confluence by adding GM2 supplemented with 0.5 mM IBMX, 0.25 μM dexamethasone, 2 μM Rosiglitazone (Sigma- Aldrich, St. Louis, Mo, USA) and 1 μg/ml insulin (Novo Nordisk, Copenhagen, Denmark). After 2 days of incubation, medium was replaced with GM2 containing 1 μg/ml insulin. Two days later, medium was replaced by GM2 and incubated for another 24 h or 5 days with the different treatments (Replacing it every 2 days). Supernatants were collected to evaluate adipokine concentration, cells were stained with Oil red O to assess intracellular neutral lipid concentration, and RNA was collected to evaluate adipogenic and lipogenic related gene transcription.

### 2.9. Myotube cell culture and differentiation

C2C12 cells were cultured in GM2 and were differentiated into myotubes using DMEM with 5.5 mM glucose, 2% Horse Serum (Sigma-Aldrich, St. Louis, Mo, USA), 2 mM glutamine, and 1% penicillin/streptomycin (GM4) for 4 days. For non-insulin-resistant cells, medium was replaced by GM4 and incubated for another 4 h with the different treatments. To develop a model of insulin-resistant cells, 4-day differentiated myotubes cells were preincubated for 2 h in DMEM with 5.5 mM glucose without FBS and supplemented with 1% BSA (Sigma-Aldrich, St. Louis, Mo, USA), and then incubated for 18 h in DMEM with 5.5 mM glucose without FBS, 1% BSA, 0.75 mM palmitate (Sigma-Aldrich, St. Louis, Mo, USA), and 200 mM insulin. C2C12 myotubes were

incubated for 4 h in 500 µl DMEM, 5.5 mM glucose, and different treatments. Supernatants were collected to evaluate glucose concentration.

### 2.10. Hepatocyte cell culture and steatosis induction

HepG2 cells were cultured in GM1. Fatty acid (FA) was prepared as FA-bovine serum albumin (BSA) complex with the ratio of oleic acid (OA): palmitic acid (PA) = 2:1. OA and PA (Sigma- Aldrich, St. Louis, Mo, USA) were dissolved in 1% BSA in low glucose DMEM by stirring for 2 h at $37^0$C. *In vitro* steatosis was induced by 0.5 mM of FA. HepG2 cells were treated with FA and treatments for 24 or 72 h. Intracellular triacylglycerol (TG) was visualized by lipid staining, and RNA was isolated for analysis of lipogenic and gluconeogenic gene expression.

### 2.11. Oxidative stress determination in J774A.1 cells

Dihydrorhodamine 123 (DHR) (Sigma-Aldrich, St. Louis, Mo, USA) was applied as qualitative marker of intracellular reactive oxygen species (ROS). J774A.1 macrophage cells were activated and treated. Cells were re-suspended in PBS (Sigma-Aldrich, St. Louis, Mo, USA) with 0.001 mM DHR, incubated for 15 min and analyzed using BD LSRFortessa™ (BD) Cytometer (excitation 488 nm, emission 530 nm).

### 2.12. Cytokine quantification in J774A.1 cells

Cytokine production in J774A.1 cells was quantified using the mouse inflammation BDTM Cytometric Bead Array (CBA) kit (50TST, BD Biosciences, San Diego, CA, USA). Flow cytometry was performed using The BD LSR Fortessa™ cell analyzer. Results were normalized to the total protein concentration. Assays were performed according to the manufacturers´ instructions. The measured cytokines were: Interleukin−6 (IL−6), Monocyte Chemoattractant Protein −1 (MCP−1) and Tumor Necrosis Factor-α (TNF-α).

### 2.13. Triacylglycerol quantification in 3T3-L1 and HepG2 cells

3T3-L1 cells were differentiated and treated for 5 days and HepG2 were fat-loaded and treated for 72 h. Cells were washed with PBS,fixed in 10% formaldehyde (Fisher Chemical, Waltham, MA, USA) at room temperature for 1 h,washed with 60% isopropanol (PanReac AppliChem, Barcelona, Spain),completely dried,stained with Oil Red-O solution (Sigma-Aldrich, St. Louis, Mo, USA) at room temperature for 30 min., and washed with water. The stain of lipid droplets was extracted with 100% isopropanol and the absorbance was measured at 492 nm in a Varioskan™ LUX multimode microplate reader (ThermoFisher Scientific, Waltham, MA, USA).

### 2.14. Adipokine quantification in 3T3-L1 cells

Leptin (ab100718) and Adiponectin (ab108785) kits (Abcam, Cambridge, UK) were used for the quantitative measurement of mouse adipokines in culture medium after cells treatment. The absorbance of each sample was measured using a Varioskan™ LUX mul-timode microplate reader (Thermo-Fisher Scientific, Waltham, MA, USA). Results were normalized to the total protein concentration. Assays were performed according to the manufacturers´ instructions.

### 2.15. RNA extraction and real-time PCR in J774A.1, 3T3-L1, and HepG2 cells

Total RNA was extracted from cells with the RNeasy kit coupled with DNase treatment for genomic DNA removal (QIAGEN, Valencia, CA, USA), and reverse transcription reaction was performed with 500 ng total RNA, 50 ng/µl random hexamers, 10 mM dNTP Mix, 20 mM Tris-HCl pH 8.4, 50 nM KCl, 2.5 mM MgCl2, 40 U/µl RNaseOut, and 200 U/µl SuperScript III RT (Invitrogen, Waltham, MA, USA), according to the manufacturers' instructions. Real-time quantitative PCR (qPCR) analyses were performed with 50 ng cDNA and 600 nM sense and antisense primers (Integrated DNA Technologies, Coral-ville, IA, USA) in a final reaction volume of 25 µl using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo-Fisher Scientific, Waltham, MA, USA) and the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The program for thermal cycling was 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 53°C, 57°C, 58°C, 59°C, 60 °C, 62 °C, 63°C or 64°C, and 30 s at 72 °C. Results were normalized to the cyclophilin B (Ppib) or Glyceraldehyde−3-Phosphate Dehydrogenase (GAPDH) expression level. For macrophages, the expression of the inflammatory marker genes Tumor Necrosis Factor (Tnf), Interleukin 1 Beta (Il1B) and Interleukin 6 (Il6) was evaluated, for adipocytes, the expression of Peroxisome Proliferator Activated Receptor Gamma (Pparg), CCAAT Enhancer Binding Protein Alpha (Cebpa), Adiponectin (Adipoq), Peroxisome Proliferator Activated Receptor Alpha (Ppara), Sterol Regulatory Element Binding Tran-scription Factor 1 (Srebf1), Acetyl-CoA Carboxylase Alpha (Acaca), Fatty Acid Synthase (Fasn), Leptin (Lep), PPARG Coactivator 1 Alpha (Ppargc1a), Uncoupling Protein 1 (Ucp1) was evaluated, and for hepatocytes, Proliferator Activated Receptor Alpha (PPARA), carnitine palmitoyltransferase 1 A (CPT1A), PPARG coactivator 1 alpha (PGC1), Protein Kinase AMP-Activated Catalytic Subunit Alpha 1 (AMPK), Sterol Regulatory Element Binding Transcription Factor 1 (SREBF1), Acetyl-CoA Carboxylase Alpha (ACC1), Acetyl-CoA Carboxylase Beta (ACC2), Fatty Acid Synthase (FASN), Peroxisome Proliferator Activated Receptor Gamma (PPARG), Phosphoenolpyruvate carboxykinase 1 (PEPCK1), and Glucose−6-Phosphatase Catalytic Subunit 1 (G6PASE) was evaluated and the relative amount of the whole mRNA was calculated using the comparative or ΔΔCt method. All the sequence-specific oligonucleotide primers (Supplementary Material - Table SI) were obtained from Invitrogen.

### 2.16. Glucose utilization in C2C12 cells

C2C12 myotubes were incubated for 4 h in GM4 with treatments. Then, 500 µl of supernatant were collected, and glucose concentration was measured using the Glucose Oxidase Assay Kit (Invitrogen, Waltham, MA, USA). To calculate glucose utilization, the remaining glucose in the culture medium after incubation with controls and Stevia extracts was subtracted from the initial amount of glucose (5.5 mM).

### 2.17. Insulin secretion in Beta-TC-6 cells

Beta-TC−6 cells were cultured in GM3 in 96-well plates and used at ~ 80% confluence. GM3 was removed and the cells were washed with PBS, and incubated for 24 h with treatments in DMEM medium with 25 mM glucose and no FBS. Cells were washed with PBS and incubated for 2 h with PBS with 2 mM glucose and the supernatant was collected (basal secretion). After 2 h, DMEM medium with 25 mM glucose without FBS was added and incubated for 30 min more. The supernatant was collected (glucose-stimulated insulin secretion "GSIS"). For all experiments, the incubation medium was collected and Insulin secreted under basal conditions and under glucose stimulation is quantified using an ultrasensitive enzyme immunoassay (Mercodia, Uppsala, Sweden). Results are expressed as percentage change in GSIS (stimulated concentration - basal concentration/basal concentration times 100).

### 2.18. Statistical analysis

Data are presented as means ± SEM. Comparisons between groups were analyzed using one-way analysis of variance (ANOVA) followed by a Dunnett post hoc test. Statistical significance was set at $p < 0.05$. Analyses were performed with the Prism 4 (GraphPad software Inc) statistical software.

**Table 1**
Extracts composition

| Compound | OBE 217 (g/100 g extract) | OBE218 (g/100 g extract) |
|---|---|---|
| **Rebaudioside A** | 32.65 | 31.16 |
| **Rebaudioside B** | 0.62 | 0.30 |
| **Dulcoside A** | 0.17 | 0.26 |
| **Rubusoside** | 0.19 | 0.23 |
| **Steviolbioside** | 0.11 | nd |
| **Stevioside** | nd | nd |
| **Rebaudioside C** | nd | nd |
| **Rebaudioside D** | nd | nd |
| **Rebaudioside F** | nd | 2.33 |
| **Total steviol glycosides** | 33.74 | 34.28 |

## 3. Results

### 3.1. Chemical characterization of AE (OBE217) and EE (OBE218) by HPLC

The steviol glycoside profile of the aqueous and ethanolic extracts used for the biological assays is shown in Table 1. The aqueous extract AE/OBE217 contained 33.74 g/100 g total steviol glycosides, mainly rebaudioside A (32.65 g/100 g), together with lower amounts of rebaudioside B (0.62 g/100 g), dulcoside A (0.17 g/100 g), rubusoside (0.19 g/100 g), and steviolbioside (0.11 g/100 g). The ethanolic extract EE/OBE218 contained 34.28 g/100 g total steviol glycosides, mainly rebaudioside A (31.16 g/100 g), together with rebaudioside B (0.30 g/100 g), rebaudioside F (2.33 g/100 g), dulcoside A (0.26 g/100 g), and rubusoside (0.23 g/100 g). Stevioside was not detectable in either extract under the HPLC conditions used. Therefore, both extracts were characterized by a high total steviol glycoside content and a strong predominance of rebaudioside A.

### 3.2. Effect of Stevia extracts on J774A.1 macrophage cell line

The effect of Stevia extracts on cell viability was determined by MTT assay (Fig. 1A). Only 800 μg/ml OBE217 produced a significant reduction of 32% in the viability of these cells. Lower concentrations of OBE217 and the tested concentrations of OBE218 produced less than a 20% reduction in viability of J774A.1 cells. For this cell line, extracts working concentrations up to 200 μg/ml were chosen. The non-toxic effects of these concentrations were confirmed by flow cytometry-based analysis using 7-AAD dye (Fig. 1B). The treatment of activated J774A.1 macrophages with OBE217 and OBE218 did not change the reactive oxygen species (ROS) production significantly (Fig. 1C).

We analyzed the anti-inflammatory effects of Stevia extracts at concentrations of 100 and 200 μg/ml. All treatments significantly decreased the transcriptional expression of Il1B, Il6, and Tnf mRNA at both concentrations (Fig. 2A–2 C). Similarly, treatment with OBE217 or OBE218 significantly reduced the production of IL−6, TNF-α and MCP1 pro-inflammatory proteins at both concentrations compared with untreated activated macrophages (Fig. 2D-2F).

### 3.3. Effect of Stevia extracts on C2C12 myocyte cell line

MTT assay determined that cell viability was not significantly reduced at the concentrations of the extracts used (Fig. 3A). To compare the effect of treatments on glucose uptake capacity, C2C12 myotubes were treated with OBE217 or OBE218, at concentrations of 100, 200 and 400 μg/ml. The effect of the extracts on glucose uptake in non-insulin-resistant C2C12 cells was to increase medium glucose uptake,

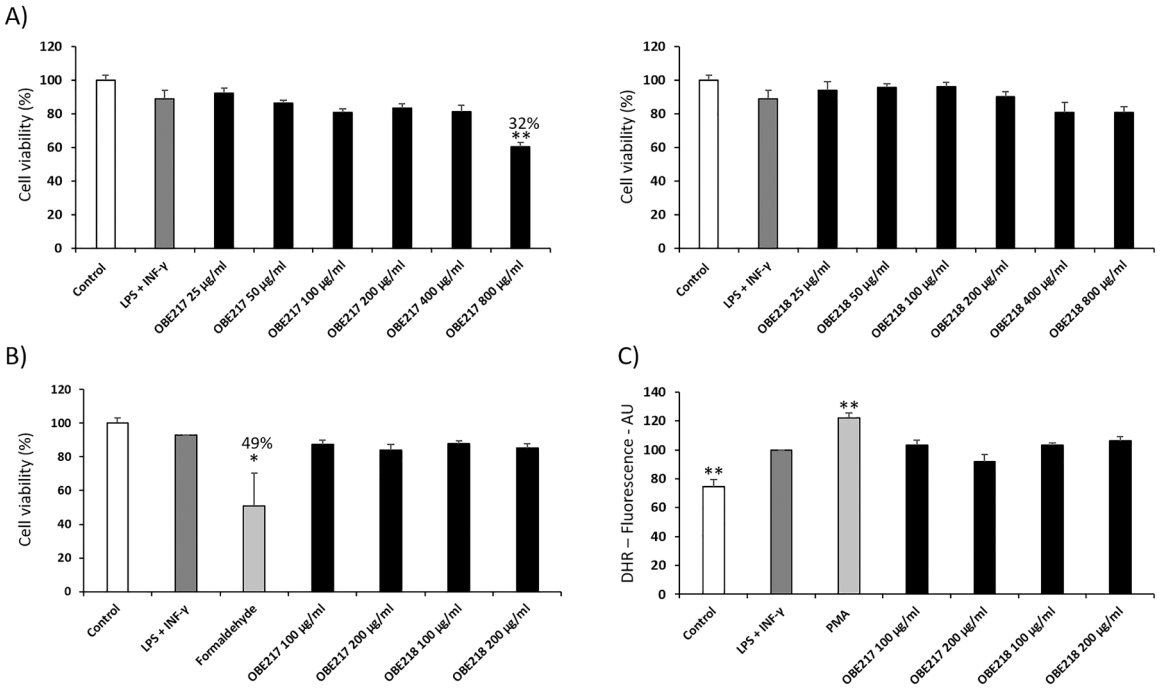


**Fig. 1.** Effect of Stevia extracts on cell viability and oxidative burst in activated J774A.1 macrophages A) J774A.1 cells were activated for 18 h and treated with various doses (0–800 $\mu$g/ml) of OBE217 and OBE218. Cell viability was measured by the MTT assay after 6 h of treatment. The percentage of viable cells was calculated by defining the cell viability without treatment as 100%. Values are expressed as mean ± SEM of three independent experiments each one performed in triplicate. B) J774A.1 cells were activated for 18 h and treated with various doses (100–200 $\mu$g/ml) of OBE217 and OBE218. Cell viability was measured by 7-AAD fluorometric assay after 6 h of treatment. The percentage of viable cells was calculated by defining the cell viability without treatment as 100%. Values are expressed as mean ± SEM of three independent experiments each one performed in duplicate. C) J774A.1 cells were activated for 18 h and treated with various doses (100–200 $\mu$g/ml) of OBE217 and OBE218. Oxidative burst was quantified by flow cytometry using Dihydro-rhodamine 123 (DHR) as a qualitative marker of intracellular reactive oxygen species (ROS) after 6 h of treatment. PMA was used as positive control. Values are expressed as mean ± SEM of three independent experiments each one performed in duplicate. * $P < 0.05$, ** $P < 0.01$, compared with LPS + IFN-γ (activated cells) (ANOVA with Dunnett́s post hoc test).

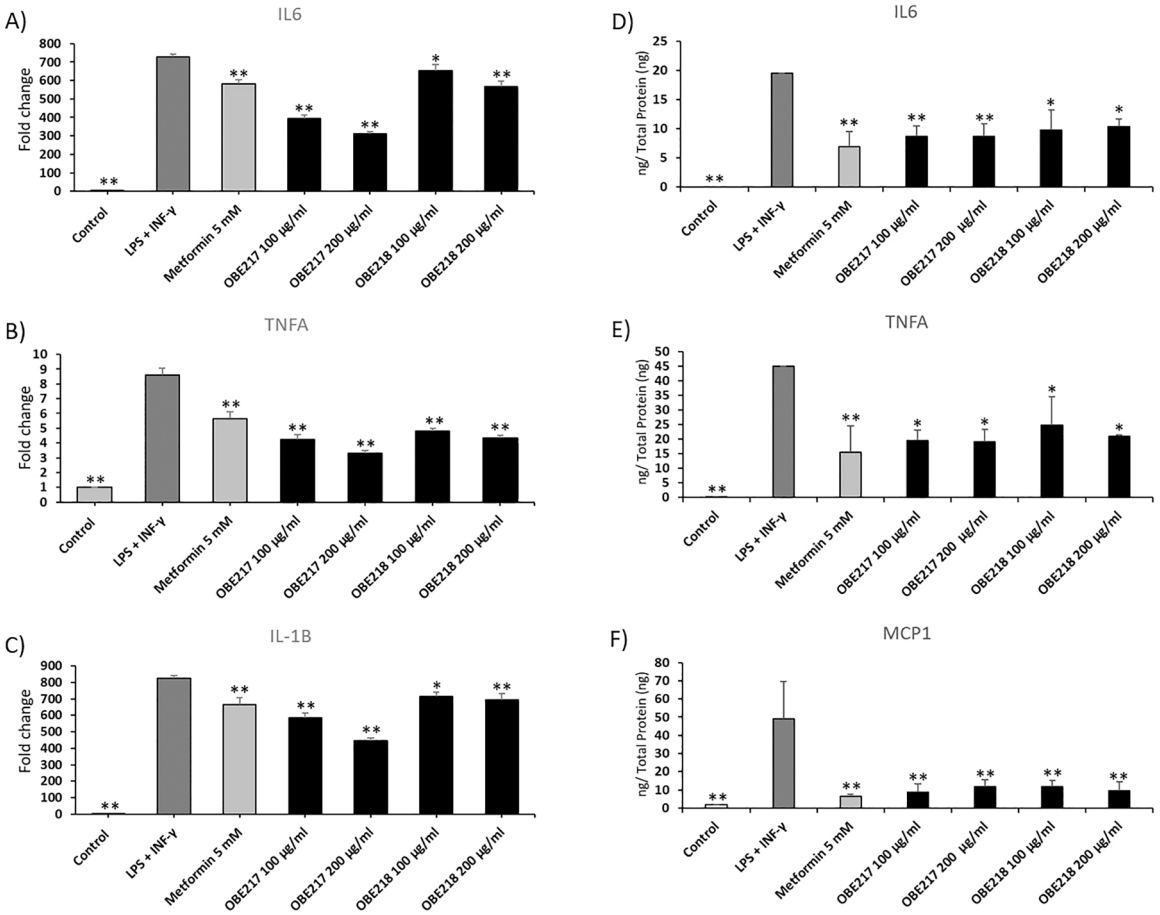


**Fig. 2.** Effect of Stevia extracts on the expression of pro-inflammatory genes and proteins in activated J774A.1 macrophages. J774A.1 cells were activated for 18 h and treated for 6 h with OBE217 and OBE218. Metformin was used as positive control. Relative expression of A) IL−6, B) TNF-α, and C) IL−1β mRNA transcripts is shown. Values are expressed as mean ± SEM of four independent experiments, normalized to the cyclophilin β gene expression. D) IL−6, E) TNF-α, and F) MCP−1 concentration in culture supernatant is shown. Values are expressed as mean ± SEM of four independent experiments. * $P < 0.05$, ** $P < 0.01$, compared with LPS + IFN-γ (activated cells) (ANOVA with Dunnett's post hoc test).

especially significant at higher concentrations (Fig. 3B). Exposure of C2C12 cells to palmitate induced insulin resistance. Fig. 3C shows that when cells were previously exposed to palmitate, glucose concentration in response to insulin was not reduced compared with that in the control (Control IR). This Fig. 3C also shows the effects of Stevia extracts on glucose uptake under conditions of insulin resistance. OBE217 and OBE218 increased glucose uptake in comparison with that of the control (Control IR). Interestingly, this response is similar to the one obtained with metformin, a drug primarily used for the treatment of T2DM.

### 3.4. Effect of Stevia extracts on HepG2 hepatocyte cell line

The effect of Stevia extracts upon HepG2 cell viability was determined by MTT assay. HepG2 cell viability was not significantly reduced at OBE217 and OBE218 concentrations used (Fig. 4A-B). The effect of the extracts treatment on HepG2 lipid accumulation is shown in Fig. 4C. OBE217 and OBE218 treatment reduced lipid content at all concentrations used. The highest concentrations used (400 µg/ml) significantly reduced the cell lipid content by 49% and 48%, enhancing the reduction observed by metformin treatment, which was used as positive control.

To verify whether OBE217 and OBE218 treatments altered hepatocyte metabolism gene expression, we used qPCR to analyze the mRNA expression levels of PPARG, CPT1A, PGC1, PPARA, SREBF1, FASN, ACC1, ACC2, PEPCK, and G6PASE (Fig. 5). The expression of PPARA, SREBF1, FASN, ACC1, ACC2, PEPCK, and G6PASE mRNA was reduced with OBE217 and OBE218 treatments at 200 and 400 µg/ml compared with that of the loaded with FFAs group (Fig. 5 D-J). Metformin, used as positive control, also downregulated the expression of the genes encoding these proteins. Expression of PPARG mRNA was significantly reduced with treatment by OBE217 and OBE218 at both concentrations but metformin treatment did not change this gene expression (Fig. 5A). Neither Stevia extracts nor metformin significantly modified CPT1A and PGC1 gene expression compared with that of the loaded with FFAs group (Fig. 5B-C).

### 3.5. Effect of Stevia extracts on 3T3-L1 adipocyte cell line

The effect of Stevia extracts upon 3T3-L1 cell viability was determined by MTT assay and viability in this cell line was similarly affected by OBE217 and OBE218 treatment, where up to 400 µg/ml of either treatment resulted in less than 20% cell cytotoxicity (Figs. 6A and 6B). For this cell line, extracts working concentrations up to 400 µg/ml were chosen. The effect of the OBE217 and OBE218 on 3T3-L1 adipocyte lipid accumulation is shown in Fig. 6C. The lipid content of cells treated with OBE217 at 100, 200 and 400 µg/ml was significantly reduced by 27%, 26%, and 33%, respectively. Only cells treated with OBE218 at 200 and 400 µg/ml had significantly reduced lipid content (28% and 23%, respectively). Metformin treatment, used as positive control, reduced fat load (29%). We assessed how treatment with these extracts at the highest concentrations affected leptin and adiponectin protein expression in 3T3-L1 adipocytes. Both extracts significantly reduced leptin and adiponectin production compared with differentiated adipocytes

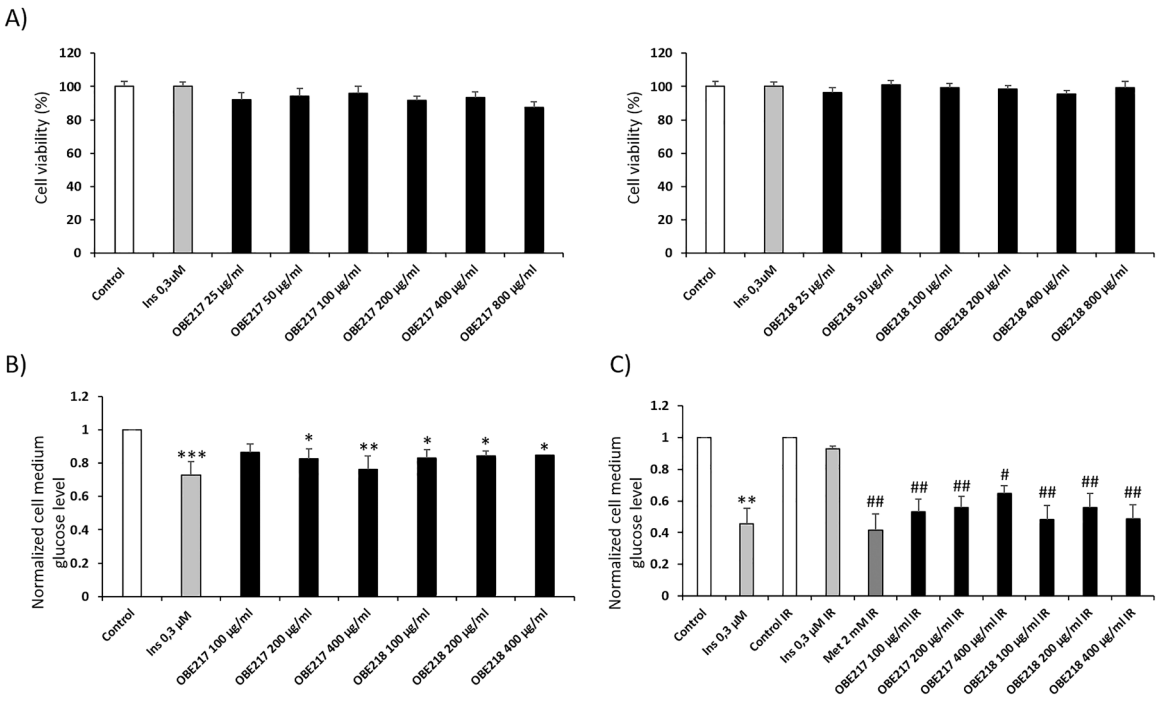


**Fig. 3.** Effect of Stevia extracts on cell viability and glucose uptake in C2C12 cells. A) Differentiated C2C12 cells were treated with various doses (0–800 $\mu$g/ml) of OBE217 and OBE218. Cell viability was measured by the MTT assay after 4 h of treatment. The percentage of viable cells was calculated by defining the cell viability without treatment as 100%. Values are expressed as mean ± SEM of three independent experiments each one performed in triplicate. B) Non-insulin-resistant C2C12 cells were treated with various doses (100–400 $\mu$g/ml) of OBE217 and OBE218. C) Insulin-resistant (IR) C2C12 cells were treated with various doses (100–400 $\mu$g/ml) of OBE217 and OBE218. B and C) Glucose was measured in cultured supernatant after 4 h of treatment by the glucose oxidase technique. Insulin (Ins) and metformin (Met) were used as controls. Values are expressed as mean ± SEM of seven independent experiments each one performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with Control; # $P < 0.05$, ## $P < 0.01$ compared with Control IR (ANOVA with Dunnett's post hoc test).

(Figs. 6D and 6E). To verify whether OBE217 and OBE218 altered adipocyte metabolism gene expression, we used qPCR to analyze the mRNA expression levels of Pparg, Cebpa, Adipoq, Ppara, Srebf1, Acaca, Fasn, Lep, Ppargc1a, and Ucp1 (Fig. 7). The expression of Fasn, and Lep mRNA was significantly reduced with OBE217 and OBE218 at both concentrations used compared with that of the untreated differentiated group (Fig. 7 G-H). Expression of Pparg, Adipoq, Srebf1 and Acaca mRNA was reduced with treatment by OBE217 at 200 and 400 µg/ml, but OBE218 significantly reduced the expression of these genes at 200 µg/ml (Fig. 7 A, C, E and F). Metformin, used as positive control, also downregulated expression of the genes encoding these proteins. No treatment significantly changed the expression of Ppara mRNA (Fig. 7D). Stevia extracts significantly upregulated the expression of Ppargc1a at the highest concentration used, and Ucp1 mRNA levels at both concentrations (Fig. 7I and J). Finally, OBE217 and metformin significantly decreased the expression of Cebpa mRNA but OBE218 upregulated the expression of this gene at the highest concentration used (Fig. 7B).

### 3.6. *Effect of Stevia extracts on Beta-TC−6 pancreatic cell line*

The effect of Stevia extracts on Beta-TC−6 cell viability was determined by MTT assay (Figs. 8A and 8B). OBE217 produced a significant decrease in the viability of these cells at concentrations higher than 50 µg/ml (Fig. 8A), and OBE218 resulted less toxic significantly reducing cell viability at concentrations higher than 200 µg/ml (Fig. 8B).

Fig. 8C shows the effect of Stevia extracts on Beta-TC−6 glucose-stimulated insulin secretion compared to basal insulin secretion. OBE217 and OBE218, although they stimulated insulin production in this cellular model, caused a significant decrease in insulin secretion compared to the control.

## 4. Discussion

Steviol diterpene glycosides, principally stevioside and rebaudiosides, are the molecules responsible for Stevia's sweetness and typically occur in leaves in the low-to-mid single-digit percentages (e.g., stevioside ~6.5–9.1%, rebaudioside A ~2.3–3.8%, with rebaudiosides D and M usually ≤0.2–0.1%) (Peteliuk et al., 2021).

In our crude extracts, HPLC showed that approximately one-third of the extract mass (≈34 g/100 g) corresponds to steviol glycosides, reflecting enrichment by extraction and concentration relative to leaf tissue. Within that fraction, rebaudioside A predominates (>90%) in both preparations (OBE217: ~97% of the glycosidic fraction; OBE218: ~91%), while minor glycosides collectively account for ~1.1% (OBE217) and ~3.1% (OBE218) of the extract. The most notable compositional difference between the extracts was the detection of rebaudioside F exclusively in the ethanolic extract (2.33 g/100 g), whereas it was not detected (under the reporting threshold) in the aqueous extract; conversely, steviolbioside was detected at a low level in OBE217 (0.11 g/100 g) but not in OBE218. These patterns are consistent with solvent-dependent recovery of minor glycosides, with ethanol favoring extraction of less polar species. For biological interpretation, the near-constancy of rebaudioside A suggests broadly similar glycosidic backgrounds between OBE217 and OBE218, whereas differences in low-abundance constituents (e.g., rebaudioside F) could contribute modestly to any assay-specific effects.

Although stevioside and rebaudioside A are commonly described as major steviol glycosides in *S. rebaudiana* leaves, stevioside was not detectable in either of the extracts analyzed here. This profile may be influenced by the particular crop material, the extraction/concentration procedures, and the analytical conditions used. Therefore, this finding is relevant for the chemical interpretation of the biological results. The effects reported here should be interpreted as responses to the complete

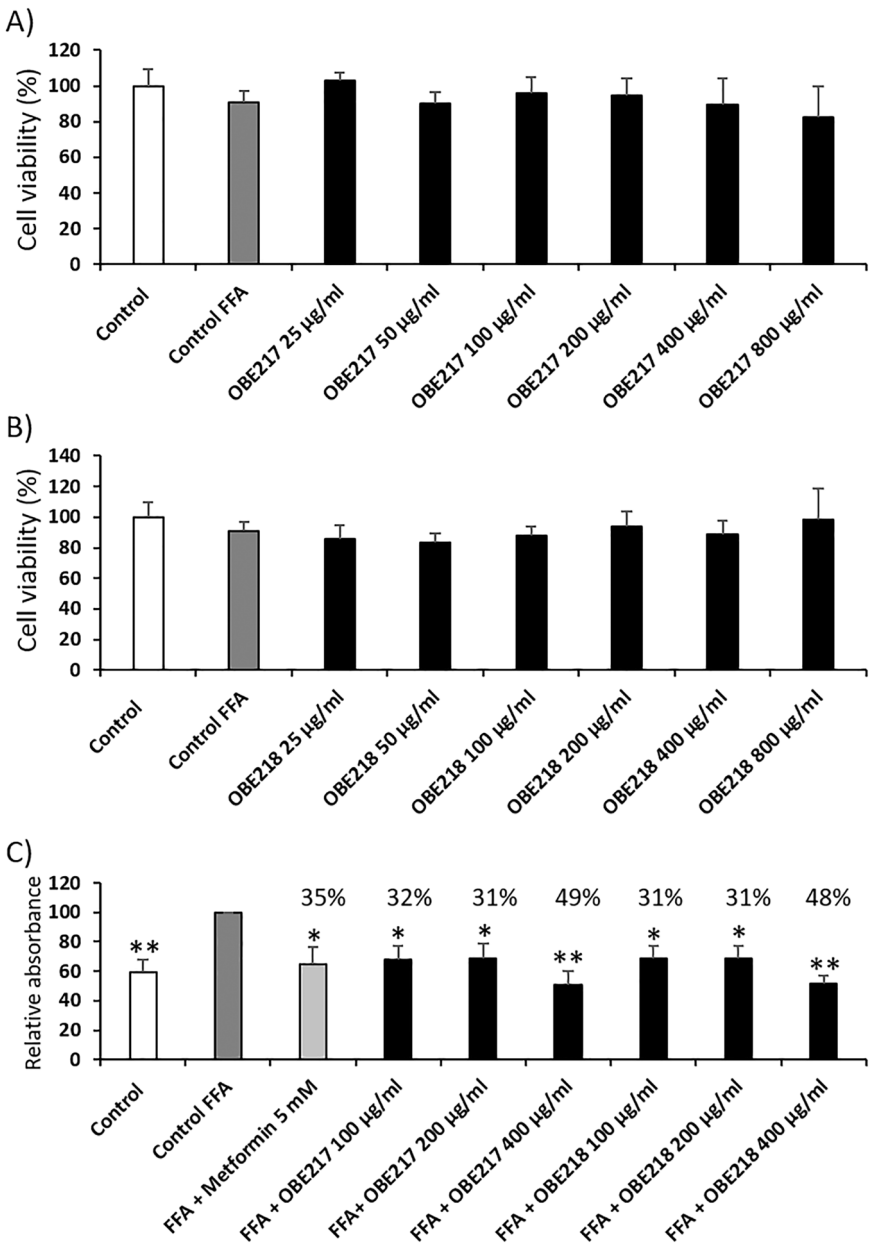


**Fig. 4.** Effect of Stevia extracts on cell viability and lipid accumulation in HepG2 hepatocytes. A)and B) HepG2 cells were treated with various doses (0–800 $\mu$g/ml) of OBE217 and OBE218. Cell viability was measured by the MTT assay after 72 h of treatment. Values are expressed as mean ± SEM of six independent experiments each one performed in triplicate. C) HepG2 cells were loaded with FFAs (oleic acid:palmitic acid/2:1) and treated with OBE217 and OBE218 for 72 h. Metformin was used as positive control. HepG2 cells were stained with oil red and lipid accumulation was quantified. Values are expressed as mean ± SEM of seven independent experiments each one performed in triplicate. * $P < 0.05$, ** $P < 0.01$ compared with FFA group (ANOVA with Dunnett's post hoc test).

OBE217 and OBE218 extract matrices, characterized mainly by high rebaudioside A content, minor steviol glycosides, and other non-glycosidic phytochemicals. Accordingly, previous studies performed with stevioside, steviol, or other isolated steviol glycosides are discussed as mechanistic background, rather than as direct evidence that the effects observed in our extracts are attributable to stevioside.

To better understand how Stevia extracts can intervene in metabolic alterations associated with obesity and diabetes, in this study, macrophage, myocyte, hepatocyte, adipocyte, and pancreatic cellular models were used to analyse the biological effect of steviol diterpenes in combination with other active molecules present in ethanolic and aqueous extracts of *S. rebaudiana* grown in the department of Antioquia, Colombia. Cell viability assays showed that high concentrations of 800 µg/ml of both extracts are not toxic to myocytes and hepatocytes, and 400 µg/ml to adipocytes (Figs. 3–6). OBE217 increased cell toxicity compared with OBE218 in macrophages and pancreatic cells, significantly reducing cell viability at higher concentrations than 400 and 50 µg/ml, respectively (Figs. 1, 8). In any case, the concentrations used of the extracts with no significant toxic effects for the cells are very high for *in vitro* studies and these data are according to other in vivo acute and chronic toxicity studies, which indicate that Stevia and stevioside have low toxicity. No mortality was reported when mice treated with acute doses of 5000 mg/kg BW of stevia crude extract, and chronic administration of 2000 mg/kg BW of stevia leaf extract to rats for 28 days lead to the survival of the animals and no pathological alterations were observed after treatment (Ruiz-Ruiz et al.,2017; Uddin et al., 2022). All these results may explain why Stevia has been consumed for over 1500 years and there have been no reports of adverse effects, demonstrating its consumption safety (Peteliuk et al., 2021).

A key factor in the development of T2DM is insulin resistance (IR)

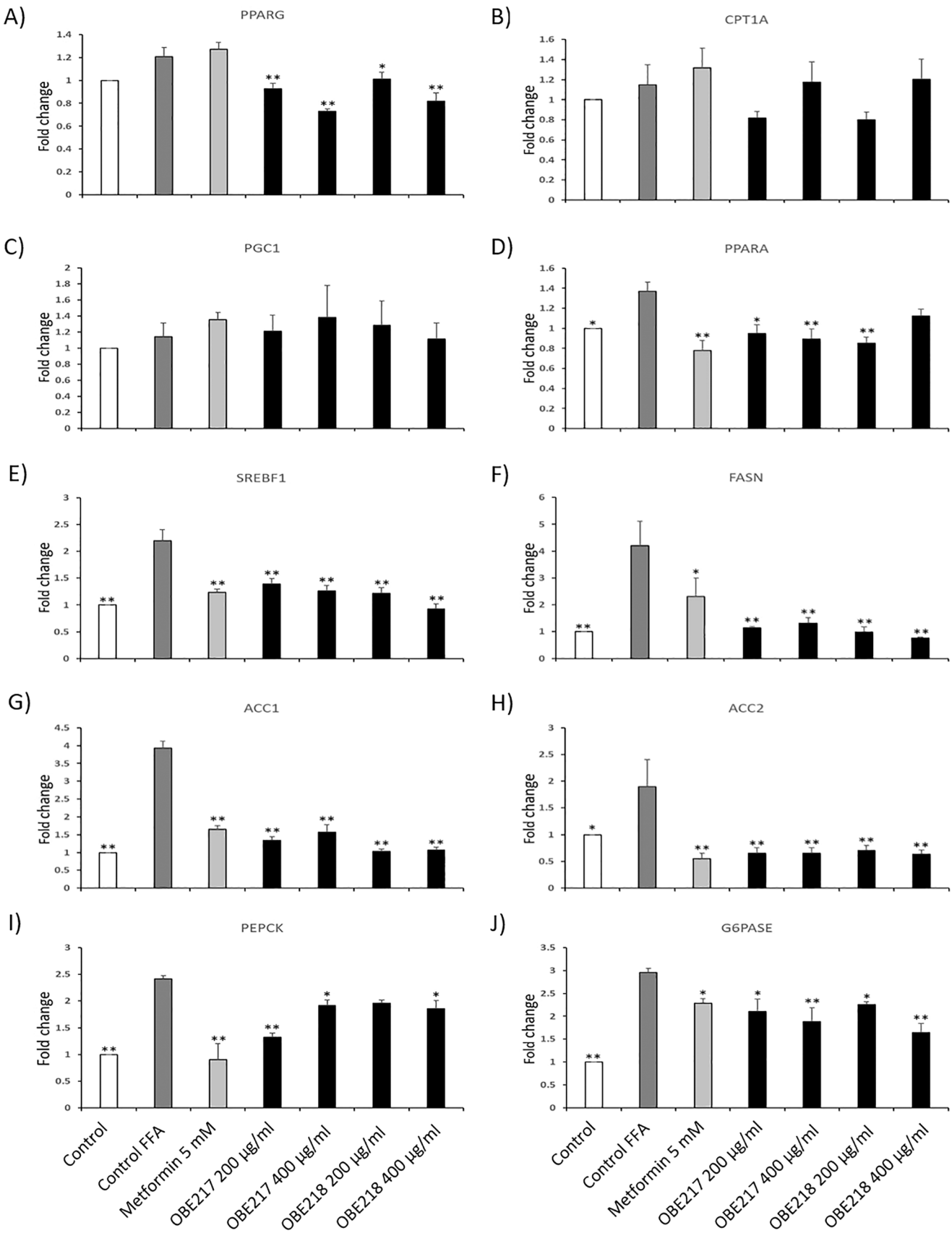


**Fig. 5.** Effect of Stevia extracts on the expression of functional genes in HepG2 cells. HepG2 cells were loaded with FFAs (oleic acid:palmitic acid/2:1) and treated with OBE217 and OBE218 for 24 h. Metformin was used as positive control. Relative expression of A) PPARG, B) CPT1A, C) PGC1, D) PPARA, E) SREBF1, F) FASN, G) ACC1, H) ACC2, I) PEPCK, J) G6PASE mRNA transcripts is shown. Values are expressed as mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs Control FFA (ANOVA with Dunnett's post hoc test).

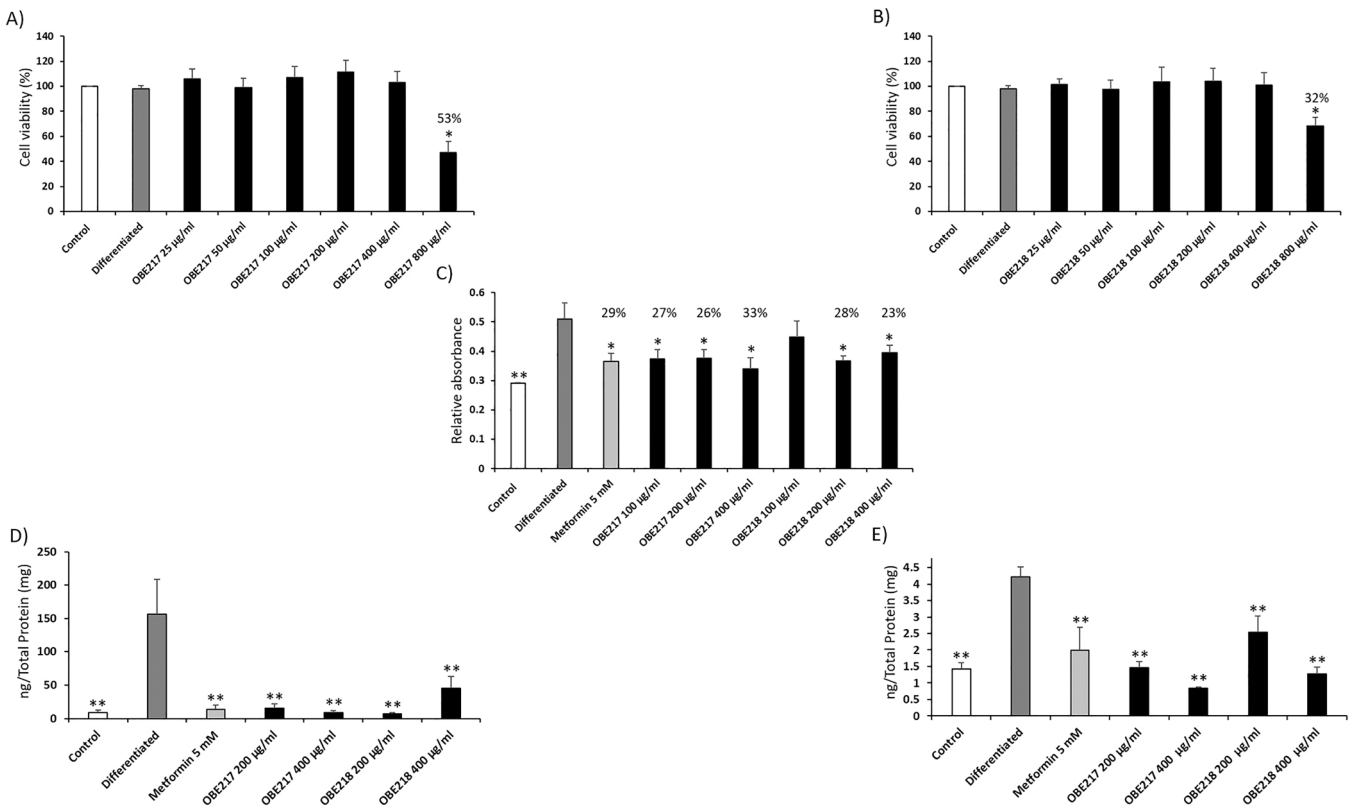


**Fig. 6.** Effect of Stevia extracts on cell viability, lipid accumulation, and protein expression in 3T3-L1 adipocytes. A and B) Differentiated 3T3-L1 cells were treated with various doses (0–800 µg/ml) of OBE217 and OBE218. Cell viability was measured by the MTT assay after 5 days of treatment. The percentage of viable cells was calculated by defining the cell viability without treatment as 100%. Values are expressed as mean ± SEM of eight independent experiments. C) Lipid accumulation was quantified after 5 days of treatment. Metformin was used as positive control. Values are expressed as mean ± SEM of nine independent experiments each one performed in triplicate. D) Amount of leptin, and E) amount of adiponectin present in the supernatant of the cells after 5 days of treatment. Values are expressed as mean ± SEM of four independent experiments each one performed in duplicate. * $P < 0.05$, ** $P < 0.01$ vs Differentiated (ANOVA with Dunnett's post hoc test).

mainly in liver, adipose tissue and muscle. The mass and the high rate of insulin-stimulated glucose transport make skeletal muscle the most important organ for whole-body glucose homeostasis. Increasing insulin sensitivity in muscle cells would help to improve blood glucose homeostasis (Di Meo et al.,2017). In our study, OBE217 and OBE218 treatments increased glucose uptake in insulin non-resistant myotubes in a manner similar to insulin effect (Fig. 3B). This effect was also observed when muscle cells become insulin resistant. Both stevia extracts significantly stimulated glucose uptake in a level comparable to metformin effect (Fig. 3C). Interestingly, regardless of the extraction method, ethanolic or aqueous, stevia extracts contain active components that produce a hypoglycaemic action. OBE217 and OBE218 also had significant downregulation of two gluconeogenic genes, PCK1 and G6PASE. This inhibition may have a favorable effect on glucose homeostasis and confirms previous data showing that stevia treatment decreases hepatic gluconeogenesis (Ray et al.,2020). These results are consistent with other studies where stevia extracts and their compounds like steviosides present antidiabetic properties. It has previously shown that aqueous and ethanolic extracts from stevia leaves decrease blood glucose levels in animal and human models with diabetes (Ahmad & Ahmad, 2018; Chowdhury et al., 2022; Ray et al.,2020; Masoumi et al., 2020; Zare et al., 2024). However, the molecular mechanisms remain unclear. Han et al. showed that Stevia extract and stevioside improve insulin resistance in the muscle of an animal model activating the AMPK/SIRT1/PGC−1α pathway (Han et al., 2023). It has also been demonstrated that stevioside stimulates the synthesis of glucose transporter 4 by activating IR/IRS−1/Akt/GLUT4 pathway (Deenadayalan et al., 2021). These mechanisms provide useful background for interpreting the increased glucose uptake observed in our cellular model. Nevertheless, because stevioside was not detectable in OBE217 and OBE218, the effects observed here should be interpreted as responses to the complete extract matrices, particularly their high rebaudioside A content together with minor glycosides and other secondary metabolites. Our present findings clearly show that the molecules present in both Colombian extracts attenuate myocyte insulin resistance and significantly improves the glucose uptake in these cells.

The effect of stevia in insulin secretion is not clarified. There are studies showing that stevia, stevioside and steviol increase insulin secretion in pancreatic cells in high glucose concentrations, however the consumption of stevia has no influence in insulin responses in healthy lean males and obese patients (Mohd-Radzman et al.,2013; Piovan et al., 2018; Samakkarnthai et al., 2018; Tey et al.,2017). We have shown that OBE217 and OBE218 induced insulin production in pancreatic Beta-TC−6 cells after glucose stimulation, but to a significantly lesser extent than in controls without stevia treatment (Fig. 8). Taking into account the effects observed in muscle and liver, this result may be beneficial considering excessive insulin secretion may lead to hypoglycemia and hyperinsulinemia is also associated with obesity and metabolic disorders (Thomas et al.,2019).

Stevia extracts treatments lead to a slight stimulation of insulin production but reduced secretion in response to glucose, which may prevent hyperinsulinemia while peripheral tissues become more insulin sensitive, increasing glucose uptake in muscle (likely via AMPK/Akt–-GLUT4) and decreasing hepatic gluconeogenesis.

Dysfunctional obese adipose tissue plays a pivotal role in the development of metabolic alterations like insulin resistance. The adipocyte hypertrophy is accompanied by increased inflammation and the infiltration of M1-type macrophages that secrete pro-inflammatory

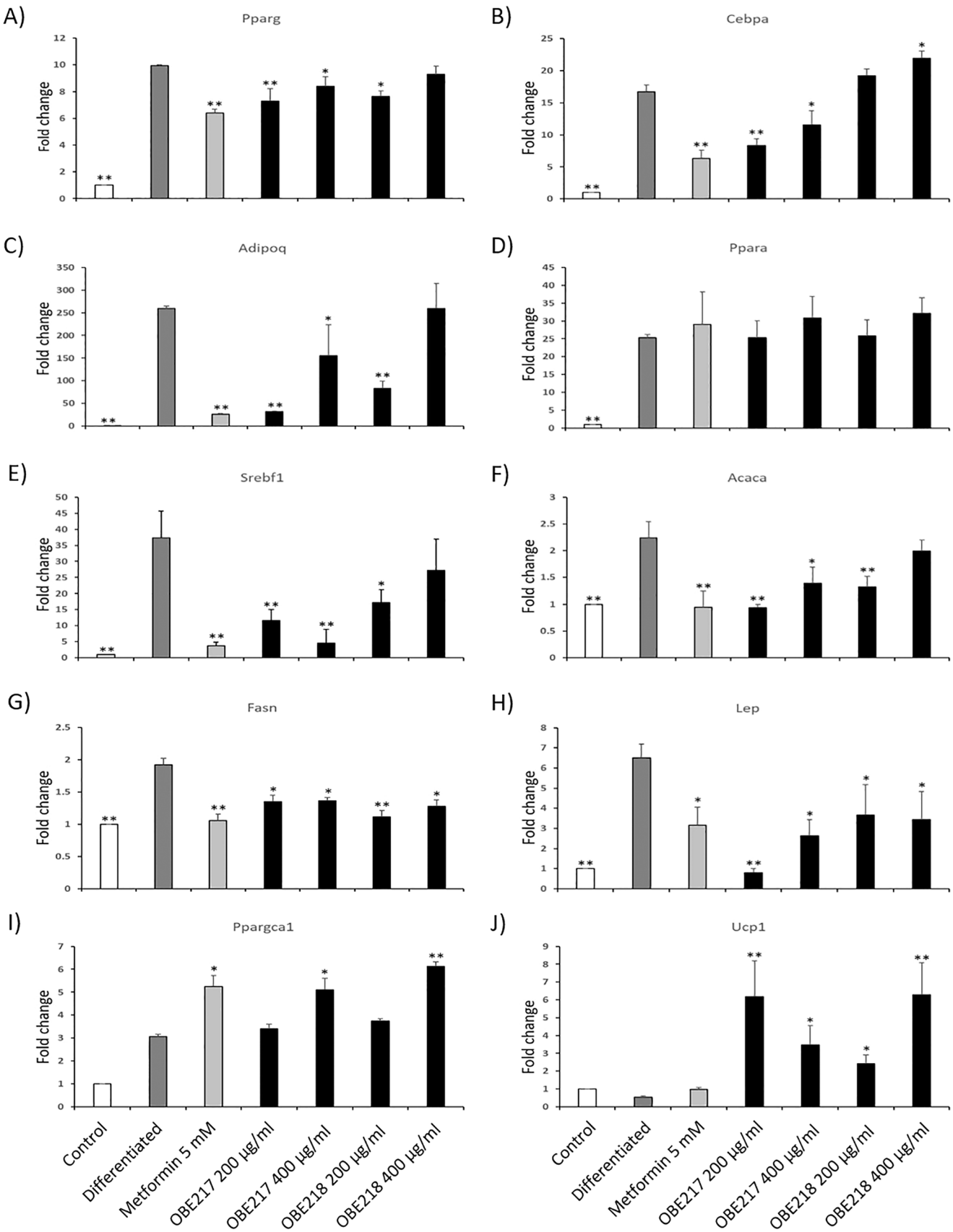


**Fig. 7.** Effect of Stevia extracts on the expression of functional genes in 3T3-L1 cells. 3T3-L1 cells were differentiated for 4 days and treated with OBE217 and OBE218 for 24 h. Metformin was used as positive control. Relative expression of A) Pparg, B) Cebpa, C) Adipoq, D) Ppara, E) Srebf1, F) Acaca, G) Fasn, H) Lep, I) Ppargc1a, J) Ucp1 mRNA transcripts is shown. Values are expressed as mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs Differentiated (ANOVA with Dunnett's post hoc test).

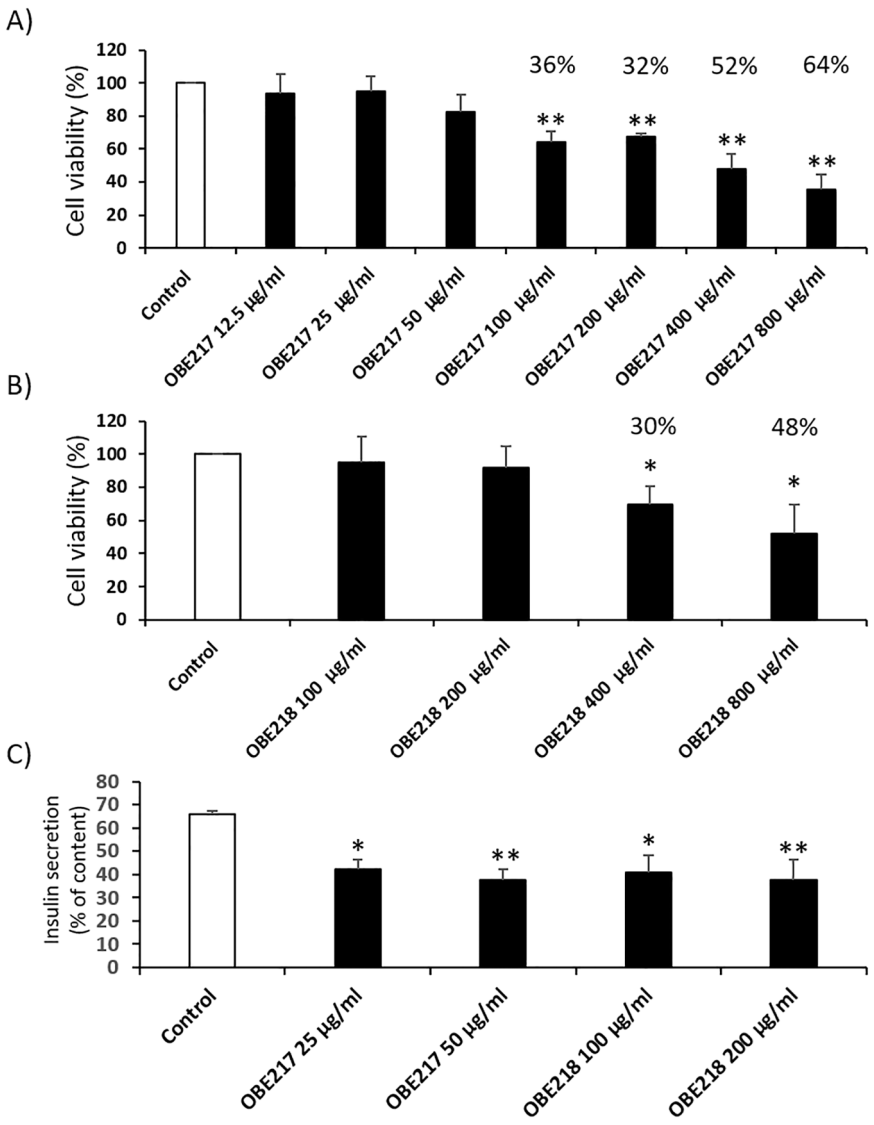


**Fig. 8.** Effect of Stevia extracts on cell viability and insulin production in Beta-TC−6 pancreatic cells. A) and B) Beta-TC−6 cells were treated with various doses (0–800 $\mu$g/ml) of OBE217 and OBE218. Cell viability was measured by the MTT assay after 24 h of treatment. Values are expressed as mean ± SEM of eight independent experiments each one performed in triplicate. C) Beta-TC−6 cells were treated with OBE217 and OBE218 for 24 h. Glucose-stimulated insulin secretion was evaluated and expressed as % of change of insulin production before and after glucose stimulation. Values are expressed as mean ± SEM of four independent experiments each one performed in triplicate. * $P < 0.05$, ** $P < 0.01$ compared with Control (ANOVA with Dunnett's post hoc test).

cytokines, a key factor for the development of insulin resistance in adipose tissue and other tissues such as the muscles and liver (Ahmed et al., 2021).

Stevia extracts have been reported to present antioxidant and anti-inflammatory properties (Sadhu et al., 2024). ROS promotes M1 macrophages and the release of pro-inflammatory cytokines (Tan et al., 2016). In this work OBE217 and OBE218 treatments did not affect macrophage ROS production (Fig. 1C), and reduced the expression of pro-inflammatory proteins, including TNF-α, IL−6, IL−1β, and MCP1 (Fig. 2). Several studies have shown the anti-inflammatory properties of stevia and its components using both *in vitro* and in vivo models. Similarly to our results, these studies have reported that stevia extracts and steviosides decreased concentrations of inflammatory markers TNF-α, IL−1 beta, IL−6 and MCP1 via downregulation of the TLR2, NF-κB, and MAPK pathways (Jeong et al., 2010; Rojas et al., 2018; Zou et al., 2020).

Stevia also presents anti-obesity properties, and several studies have investigated the relationship between stevia consumption and weight loss, influencing satiety and modify fatty acid metabolism (Dharani et al., 2024; Ray et al.,2020; Peteliuk et al., 2021). In our study, OBE217 and OBE218 reduced lipid content in 3T3-L1 cells, and they have a similar effect as metformin (Fig. 6C). These results are according to the reduction of lipid accumulation and TG content observed by the treatment of stevia compounds like stevioside and steviol in this cellular model (Kurek et al.,2022; Park, Sharma, et al.,2022). We have shown that the whole extracts and metformin, used a positive control, also reduced the production of leptin and adiponectin (Figs. 6D-E, 7C-H). Leptin production is associated with obesity and adipose tissue hypertrophy and inflammation. Leptin acts directly on macrophages to increase their pro-inflammatory cytokine production, and there is an association between plasma leptin concentrations and insulin resistance. By contrast, adiponectin has anti-inflammatory properties and is directly related to insulin sensitivity. However, it has been described that overexpression of adiponectin in 3T3-L1 fibroblasts promotes their differentiation to adipocytes and accumulation of larger lipid drops (Fu et al.,2005; Gunturiz Albarracín & Forero Torres, 2020). In this cellular model the reduction of the expression of both adipokines has beneficial effects and the molecules responsible for this inhibitory effect may be rebaudioside A, as previously documented in 3T3-L1 model (Kurek

et al., 2022).

Our study aimed to determine the effect of stevia extracts on the expression of genes involved in adipogenesis and lipid metabolism. Steviol and stevioside have been previously reported active compounds inhibiting the expression of Pparg, Cebpa, Srebf1 and Fasn (Kurek et al., 2022; Park, Baek, et al., 2022). OBE217 significantly downregulated the expression of the adipogenic transcription factors Pparg, Cebpa, and Srebf1, and the lipogenic genes Fasn, and Acaca (Ayala-Sumuano et al., 2011; Schouwink et al.,2025). The effect of OBE218 was not as strong as the aqueous extract in the modulation of the expression of these genes. In any case, 3T3-L1 intracellular fat accumulation reduction may be partially explained by modification of the expression of these genes by the stevia extracts (Fig. 7). Both extracts also upregulated the expression of mRNA of Ppargc1a, and Ucp1, and did not affect the expression of Ppara. These genes are associated with oxidation of fatty acids and are highly expressed in brown fat. The upregulation of Ucp1 mRNA is clearly associated with the brown fat cell phenotype (Gong et al., 2024), and Ppargc1a expression is reduced during adipocyte differentiation and cooperates with Ppara to control lipid oxidation and thermogenesis in the brown adipose tissue. The Ppargc1a expression regulation by OBE217 and OBE218 may also influence adipocyte differentiation and maturation (Corrales et al.,2018).

In a similar way to adipocytes, OBE217 and OBE218 reduced the liver concentration of triglyceride with effects comparable to those produced by metformin treatment (Fig. 4C). These results are consistent with in vivo studies when a stevia supplementation led to a significant reduction in serum and liver concentration of triglyceride (J. E. Park & Cha, 2010). These effects may be explained by the action of stevioside, steviol and rebaudioside A, compounds which have been reported to ameliorate hepatic steatosis (Holvoet et al., 2015; Park, Sharma, et al., 2022).

The analysis of the expression of hepatic genes involved in lipid and carbohydrate metabolism may explain this beneficial effect. Both extracts significantly downregulated the expression of the adipogenic transcription factors PPARG and SREBF1, and the lipogenic genes FASN, ACC1, ACC2 (Fig. 5). These data are in accordance with the reduction of the expression of these genes in db/db mice livers treated with stevia and stevioside (M. Park, Sharma, et al.,2022). We also have found that stevia extracts inhibit the expression of PPARA and have no significant effect on the expression of CPT1A and PGC1 genes. PPARA is activated by lipid species like fatty acids and together with CPT1A and PGC1 the upregulation of these genes are associated with an increase in fatty acid oxidation (Fang et al., 2022; Kersten & Stienstra, 2017). In view of these results, the reduction of the hepatocyte fat load by stevia extracts may be explained by an inhibition of lipogenesis more than an activation of fatty acid oxidation.

These findings suggest that OBE217 and OBE218 probably act at the level of transcriptional regulation suppressing inflammatory transcriptional programs in macrophages, not by scavenging ROS in these cells, reducing cytokine production and thereby systemic inflammation and insulin resistance, and suppression of lipogenic genes in adipocytes and hepatocytes decreased lipid synthesis, effect enhanced with a more thermogenic phenotype in adipocytes.

Our work provides evidence that stevia extracts from Antioquia, Colombia have anti-inflammatory and hypolipogenic activities, and can modulate glucose uptake. The mix of glycosides and secondary metabolites present in OBE217 and OBE218 may allow synergism or complementary effects that could act on different cell types and metabolic pathways. OBE217 and OBE218 have the advantage of lower toxicity and may improve metabolic disorders associated with obesity and T2DM. Given their chemical profile, the observed biological effects are most appropriately interpreted as responses to Stevia extract matrices highly enriched in rebaudioside A, rather than as effects attributable to a single steviol glycoside. Further research is necessary to study the composition of the extracts and to confirm their beneficial effects in vivo models.

## CRediT authorship contribution statement

**Cardona Helen:** Methodology, Formal analysis, Data curation. **Mora Valentina:** Methodology, Formal analysis, Data curation. **Peláez Jaramillo Carlos:** Project administration, Funding acquisition. **Nora Restrepo-Sánchez:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Norman Balcazar:** Writing – review & editing, Conceptualization. **Muñoz Diana:** Methodology, Formal analysis, Data curation. **Sergio Acin:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.foohum.2026.101320.

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