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Native cacao from southern Mexico: Polyphenol profiling and its impact on inflammation and lipid metabolism modulation

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ABSTRACT

Background: Cacao (*Theobroma cacao* L.) is well known for its polyphenolic profile, which confers antioxidant and anti-inflammatory properties. This investigation provides remarkable evidence on the functional potential of the native Criollo cacao variety *Rojo Samuel* (RS), a poorly characterized cultivar from southern Mexico, particularly regarding its effects on macrophage-mediated inflammation and lipid metabolism.

Methods: Phenolic compounds were extracted, quantified, and characterized from cacao beans. TNFα expression and nitric oxide production (NO) were measured in RAW 264.7 macrophages treated with cacao phenolic extracts. The lipid accumulation in adipocytes (3T3-L1) was evaluated using Oil Red O stain and triglyceride accumulation during the differentiation process. PPARγ and fatty acid synthase (FAS) expression were determined by Western blot in mature adipocytes treated with native cacao extracts.

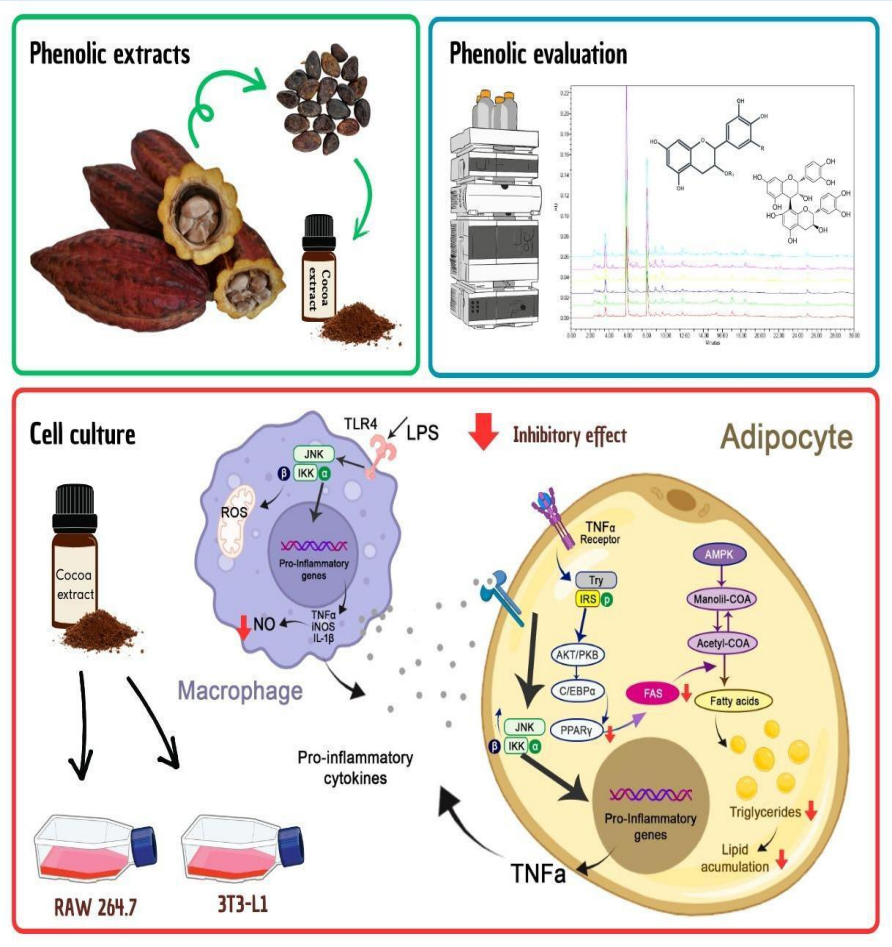
Results: In RAW 264.7 macrophages, treatment with cacao phenolic extracts significantly reduced NO production by 20-75%; however, TNF-α expression was not significantly affected by the treatment. In 3T3-L1 adipocytes, treatment with

150 µg extract/mL during adipocyte differentiation significantly decreased lipid accumulation (20.6%), intracellular triglyceride content (41.3%), PPARγ expression (81.2%), and FAS expression (40.2%). These effects are likely attributable to the presence of a variety of phenolic compounds in the cacao extracts, including epicatechin, catechin, procyanidins, and others.

Novelty of the Study: Unlike prior research that focused primarily on cacao for industrial applications, this study demonstrates that phenolic extracts from the Criollo cacao variety Rojo Samuel (RS) exhibit antioxidant properties, modulate macrophage-mediated inflammation, and influence lipid metabolism in adipocytes. These findings highlight the potential of this native variety as a valuable source of functional food substances.

Conclusion: Phenolic compounds from RS cacao showed biological activity relevant to inflammation and lipid metabolism regulation, in addition to their antioxidant effects. Furthermore, this study highlights the importance of this native underexplored cacao variety as a promising source of functional food substances.

Keywords: Criollo cacao, polyphenols, inflammation, lipid metabolism.



Graphical Abstract: Native cacao from southern Mexico: Polyphenol profiling and its impact on inflammation and lipid metabolism modulation

INTRODUCTION

Metabolic diseases, such as obesity, are on the rise. The WHO reported that 43% of adults over 18 were overweight, and 16% were obese [1]. Obesity is considered an inflammatory disease involving complex cellular and molecular processes [2,3]. Therefore, cellular responses can dysregulate, disrupt physiological homeostasis, and contribute to the development of chronic inflammatory diseases [3].

Phenolic compounds have garnered considerable attention due to their strong antioxidant activity and anti-inflammatory properties, demonstrated in the management of hypertension, metabolic disorders, inflammatory diseases, infections, and neurodegenerative diseases [4]. Among cacao varieties, Criollo cacao is reported to contain higher concentrations of phenolic compounds when roasted at low temperatures [5,6]. Furthermore, it has been noted that fermentation significantly influences the polyphenolic profile of beans: unfermented beans contain higher concentrations of oligomeric proanthocyanidins, whereas fermented beans accumulate compounds, such as phenolic glycosides, through enzymatic and microbial transformations [7]. These observations highlight the relevance of investigating unfermented Criollo cacao beans as an abundant source of polyphenols that may have advantageous health-promoting properties.

In vitro studies have shown that flavonoids in Forastero cacao from Malaysia decreased the production of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β), as well as reactive oxygen species (ROS) and reactive nitrogen species (RNS) in LPS-stimulated macrophages. [8]. Similarly, polyphenols from cacao shell extract reduced the inflammatory interaction between RAW264.7 macrophages and 3T3-L1 adipocytes, preserving mitochondrial function and insulin sensitivity in adipocytes [9]. In addition, although the cacao variety was not specified, a study reported that cacao phenolic extract enhanced leptin production and inhibited lipid accumulation in 3T3-L1 preadipocytes [10].

This study aimed to evaluate the content and profile of phenolic compounds in native Criollo RS cacao and determine their effects on macrophage-mediated inflammation and lipid metabolism in adipocytes.

MATERIALS AND METHODS

Material: Hexane (95%), acetone ACS (≥99.5%), methanol ACS and HPLC (≥99.8%), and ethanol (89-91%) were purchased from Fermont (Monterrey, MX). Acetonitrile (99.5%) was purchased from Karal (León, Guanajuato, MX). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Plant material: Samples of native Criollo cacao, RS variety, were donated from INIFAP Rosario Izapa Experimental Field, Tuxtla Chico, Chiapas, Mexico. Cacao beans were cleaned and dried at 50°C for 24 hours in a convection oven (VenticellEcoline, MMMGroup, DEU) before being ground. The samples were kept frozen (-18°C) until analysis.

Phenol extraction and quantification: Cacao bean powder was defatted with hexane (1:10 *m*/*v*) [11]. Phenolic extracts were prepared according to Oracz et. al. [12] with slight modifications. Two grams of defatted sample were extracted three times with 10 mL of acetone-water-acetic acid (70:29.5:0.5 *v*/*v*/*v*) for 30 minutes in the ultrasonic bath at 40 kHz (CP08895, Cole-Parmer, Illinois, USA) and centrifuged for 15 minutes (1500 rpm, 4°C). Acetone was removed using a rotary evaporator at 40°C under vacuum conditions, and the aqueous extract was adjusted to 10 mL with distilled water. Extracts were frozen at -18°C, lyophilized for cell assays, and stored at -20°C.

Total phenols (TP), flavonoids (TF), and condensed tannins (TC) were quantified using Folin-Ciocalteu, aluminum chloride, and vanillin assays, respectively [13], using a UV-Visible spectrophotometer (Infinite M200 Pro, TECAN, Männedorf, Switzerland). The standard curves of gallic acid (R^2 = 0.993), rutin (R^2 = 0.998), and catechin (R^2 = 0.996) were used (n=2).

Antioxidant capacity: The bioactivity of RS extract was evaluated through its ability to scavenge free radicals (DPPH and ABTS) and neutralize reactive oxygen species (ROS) (ORAC). For the DPPH assay, RS extract (1:200, methanol) was mixed 1:1 with DPPH+, and absorbance was read at 515 nm after 30 min. In ABTS assay, it was measured at 734 nm after 24 min [13]. The ORAC assay was performed by incubating the reaction for 10 minutes at 37°C, and fluorescence was measured at an excitation of 485 nm and an emission of 528 nm at 2-minute intervals for 3 hours [14]. The results are expressed as µM Trolox equivalents per milligram of extract (n=2).

UHPLC characterization: Phenolic compounds were identified according to Contreras et al [15] using UHPLC-DAD (Aquity Arc, Waters) with a C18 column (2.7 µm, 4.6 × 150 mm; Cortecs, Waters, Milford, MA, USA).

Cell culture: The RAW 264.7 macrophage (TIB-7) and 3T3-L1 preadipocyte (CL-173) cell lines were purchased from ATCC (Manassas, VA, USA). Cell lines were maintained in Dulbecco's modified Eagle culture medium (DMEM) (Biowest, Cat. L0104-500, Nuaillé, FR) supplemented with 10% FBS/NCS, respectively, and 1% antibiotics (penicillin 10,000 U/mL/streptomycin 5 mg/mL), and incubated at 37°C in a humidified atmosphere with 5% CO_2.

Adipocyte differentiation: Preadipocytes were treated for 72 h with differentiation medium 1 (DM1) containing DMEM supplemented with 10% FBS, 1% antibiotics (penicillin/streptomycin), 0.25 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 µg/mL insulin, and 2µM rosiglitazone (Day 3). The medium was subsequently replaced with differentiation medium 2 (DM2), which contained DMEM supplemented with 10% FBS, 1% antibiotics, and 1 µg/mL insulin (Day 6) and incubated for 72 h. Then, adipocytes were maintained in maintenance medium (MM) containing DMEM supplemented with 10% FBS and 1% antibiotics [16].

Cell viability: Macrophages RAW 264.7 [2×10^4 cells/well, 96-well plates] and Adipocytes 3T3-L1 [6,000 cells/cm^2, 96-well plates] were treated with RS extract (100-1000 µg/mL) or catechin standard (CA) (100-1000 µM/L) for 24 hours. Cell viability was measured using the MTT assay with the Cell Proliferation Kit I (Roche, Mannheim, AL) according to the manufacturer's instructions. [8-10].

NO production: Macrophages RAW 264.7 [2×10^4 cells/well, 96-well plates] were treated with RS extract (25-500 µg extract/mL) and catechin (25-500 µM/L) for 4 hours and stimulated with LPS (1 µg/mL) for 24 hours. NO was determined by mixing the Griess reagent with the supernatant (1:1) for 15 minutes [17].

TNFα expression on macrophages RAW 264.7: Macrophages RAW 264.7 [6×10^5 cells/well, 6-well plate] were treated for 4 hours with the RS extract (100 µg/mL) or catechin (100 µM) and stimulated with 1 µg/mL of LPS for 24 hours. Cell lysates were prepared to determine TNFα expression by Western blotting. [18].

Lipid and triglyceride accumulation on adipocytes 3T3-L1: Preadipocytes were seeded [6,000 cells/cm^2, 24-well plates] and differentiated upon confluence. Then, they were treated with RS extract (50-150 µg/mL) or CA (50-150 µM) from day 6 to 13-15; treatments were applied at each medium change. Oil red staining (ORO) was performed to assess lipid accumulation [17]

PPARγ and FAS expression on mature adipocytes 3T3-L1: Cells were seeded [6,000 cells/cm^2, 6-well plates] and were differentiated. Cells were treated from day 6 until complete differentiation with RS extract (50 and 150 µg/mL) and catechin (50 and 150 µM). PPARγ and FAS expression were evaluated in cell lysates using Western blotting [18].

Western Blot: Protein expression of molecular markers was assessed by Western blot [18].

Statistical analysis: All the measurements were analyzed using STATGRAPHICS Centurion XVI.I software (Statgraphics Technologies Inc., The Plains, Virginia, USA).

RESULTS

Phenolic compounds and antioxidant activity of RS extract: The phenolic extract of Rojo Samuel cacao showed a high content of condensed tannins (159.3 ± 14.99 mg CA/g), total phenolic compounds (43.05 ± 6.22 mg GAE/g), and flavonoids (2.26 ± 0.00 mg RE/g). Among the most abundant compounds were epicatechin (3.17 ± 0.28 mg/g), sinapic acid, catechin, and epigallocatechin, while others such as gallic acid and rutin were present in traces (Table 1). These results suggest that this Criollo variety is a valuable source of bioactive compounds with potential for nutraceutical ingredients. The antioxidant capacity of the extract was detailed by DPPH, ABTS, and ORAC assays (Table 1).

Table 1. TPC, TF, TC, phenolic profile by UHPLC, and antioxidant capacity of RS

Compound	**Concentration**
TP (mg GAE/g.)	43.05 ± 6.22^a
TF (mg RE/g)	2.26 ± 0.00 ^{a}
TC (mg CA/g.)	159.30 ± 14.99^a
mg / g extract	
Epicatechin	3.17 ± 0.28^a
Chlorogenic acid	1.72 ± 0.00^b
µg / g extract	
Catechin	79.79 ± 7.46^b
Sinapic acid	189.57 ± 22.51^a
Syringic acid	31.91 ± 3.43^b
Naringin	58.17 ± 14.62^a
Epigallocatechin	54.70 ± 1.02^{ab}
Gallic acid	<2.70
Caffeic acid	<3.01
Rutin	<2.05
Quercetin-3-Galactoside	<2.27
Quercetin	ND
Ferulic acid	ND
Kamferol-3-Glucosido	<1.25
Antioxidant capacity (µM TE*/g)*	
DPPH	2.78 ± 0.11
ABTS	105.42 ± 2.01
ORAC	13 772.16 ± 1 019.47

Data are reported as mean ± standard deviation (n=2). Different letters per row express significant differences based on ANOVA and Tukey's multiple range test ($p<0.05$).

Cytotoxic measurement of RS extract on macrophages RAW 264.7 and adipocytes 3T3-L1*:* RS extract was applied to 3T3-L1 macrophages and preadipocytes to evaluate their cytotoxicity (Figure 1) [19]. In RAW 264.7 macrophages, concentrations of 100 and 500 µg/mL showed no cytotoxicity at 2 hours, but reduced cell viability at 24 hours (Figure 1A). In 3T3-L1 preadipocytes, cell lysis was observed at 1000 µg/mL, and morphological changes indicative of apoptosis were observed at 500 µg/mL after 48 hours (Figure 1B).

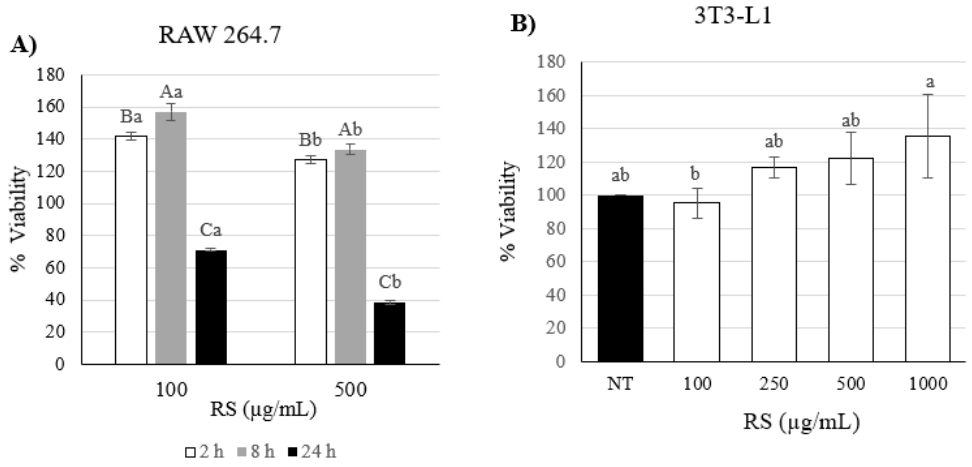


Figure 1. Cell viability assessed by MTT in RAW 264.7 (**A**) and 3T3-L1 (**B**). Data expressed as mean ± SD (n=3). Letters indicate significant differences by ANOVA and Tukey's test ($p < 0.05$); uppercase letters indicate times, lowercase letters indicate concentrations.

Effect of phenolic extracts on NO production and TNFα expression on macrophages RAW 264.7: Macrophage infiltration and the production of NO and TNFα contribute to chronic inflammation in metabolic diseases [20]. In RAW 264.7 macrophages, RS extract significantly reduced NO production (20.47–75.78%), whereas catechin showed greater inhibition at 250 µM/mL (86.03%) (Figure 2A). The IC50 of RS (221.93 ± 13.37 µg/mL) was lower than that of CA (303.77 ± 7.21 µM/mL). However, neither treatment significantly affected TNFα expression upon LPS stimulation (Figure 2B).

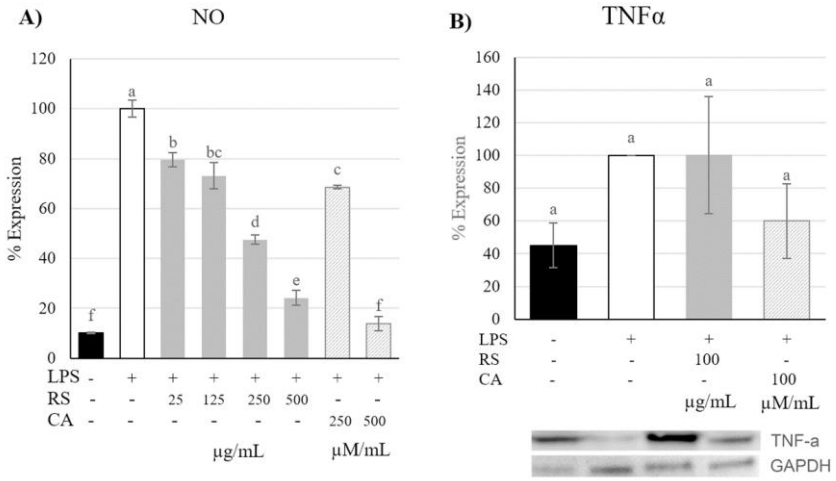


Figure 2. Effect of RS extract and CA on NO **(A)** and TNFα **(B)** on RAW 264.7. Different letters express significant differences (ANOVA, $p<0.05$, Tukey, n=3).

Effect of phenolic extracts on lipid and triglyceride accumulation in adipocytes 3T3-L1: Differentiated adipocytes were treated with RS (50–150 µg/mL) and CA (50–150 µM/mL) from day 6 to evaluate lipid and TG accumulation. ORO staining confirmed cell differentiation (Figure 3), with more than 80% conversion in the NT group. Treatment with RS at 150 µg/mL significantly reduced lipid accumulation, whereas CA showed minor effects. Subsequent quantification (Figure 4) confirmed the RS-induced decrease in lipids and TG during differentiation.

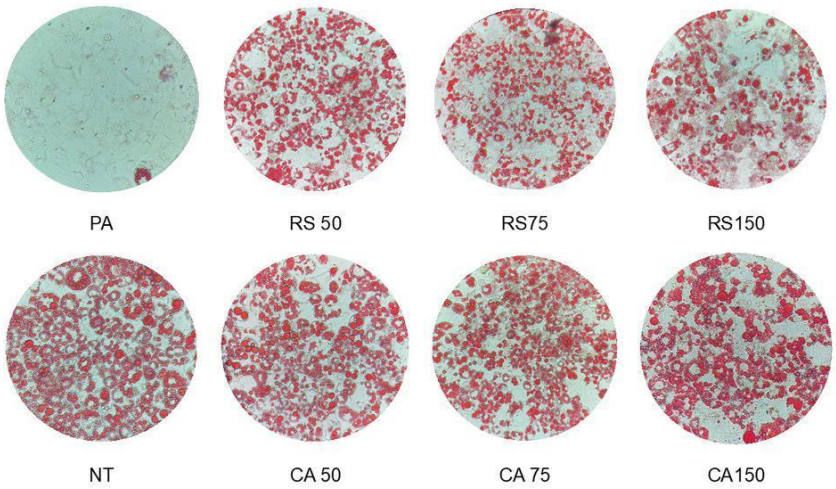


Figure 3. Lipid accumulation on adipocytes treated with RS (50-150 µg/mL) and CA (50-150 µM/mL). Lipid accumulation, assessed with ORO (Figure 4A), was significantly reduced by RS at 150 µg/mL (20.56%) and CA at 50 µmol/L (17.28%), with no relevant effects at lower concentrations. Regarding triglycerides (Figure 4B), only RS at 150 µg/mL significantly reduced their content (54.40%) compared to the control; the other treatments showed no significant differences.

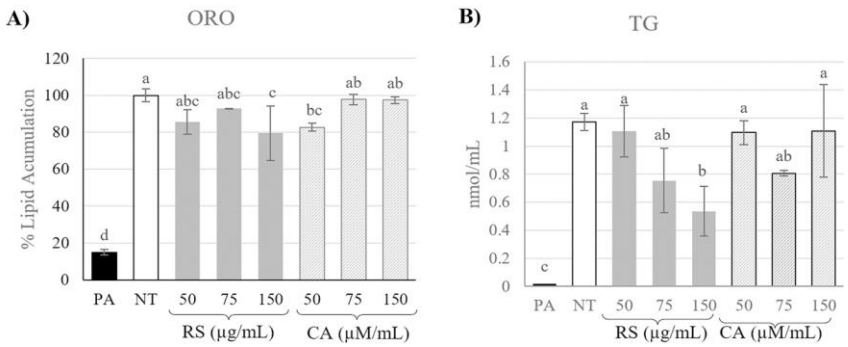


Figure 4. Effect of RS and CA on lipid **(A)** and triglyceride **(B)** accumulation during differentiation. Different letters indicate significant differences (ANOVA and Tukey, $p < 0.05$).

Effect of phenolic extracts on PPARγ and FAS expression on mature adipocytes 3T3-L1: PPARγ regulates key genes involved in fat accumulation and insulin sensitivity in 3T3-L1 adipocytes, including FAS [21]. RS extract significantly inhibited PPARγ expression by 81.17% and 54.01% at 150 and 50 µg/mL, respectively, whereas CA showed no significant effect at any concentration (Figure 5A). Similarly, only RS at 150 µg/mL reduced FAS expression (40.20%) (Figure 5B). No other treatments showed significant differences versus the untreated control (NT).

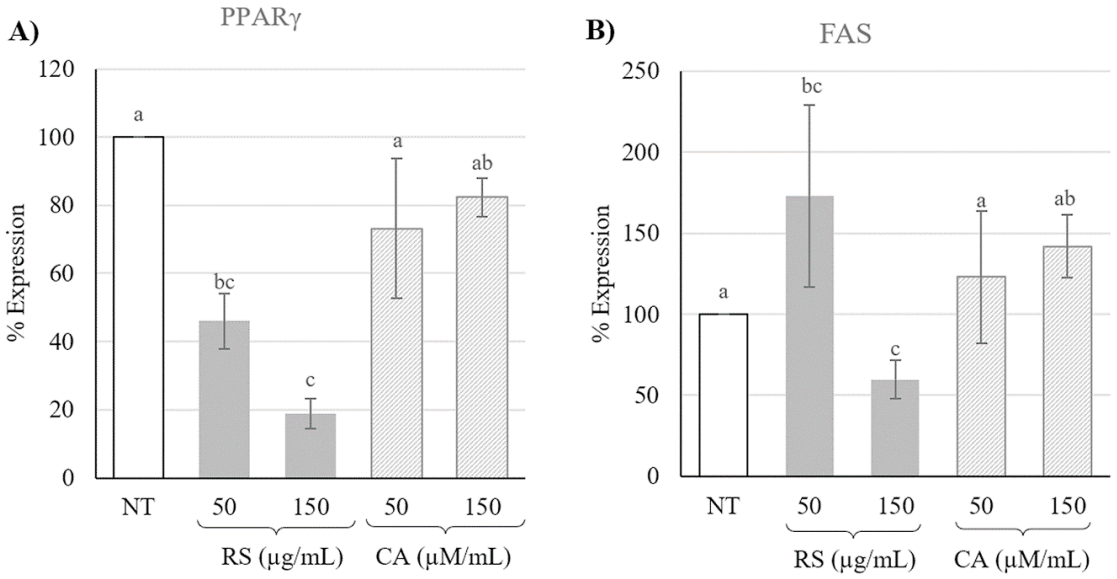


Figure 5. PPARγ (A) and FAS (B) expression on adipocytes. Different letters per row express significant differences based on ANOVA and Tukey's multiple range test ($p<0.05$).

DISCUSSION

Quantification and characterization of the phenolic compounds of RS phenolic cacao extract: The total phenolic content (TPC) of RS extract matched values for Criollo cacaos from Soconusco [22] and exceeded previous reports for the same variety [23]. Despite Criollo's typically lower tannin levels, RS concentrations were comparable to Forastero beans from Ghana [24] and higher than CNN51, ICS, and TSH clones [25], likely due to climate, processing, or genetic factors [22].

Among the identified compounds, epicatechin and catechin predominated, both associated with their beneficial effects on insulin sensitivity, lipid profile, and digestive enzyme inhibition [26–30]. Other bioactive phenolic compounds, such as sinapic acid, chlorogenic acid, and naringin, were also identified, although some flavonoids were present only in trace amounts or were not detected.

Antioxidant capacity: The RS extract demonstrated a higher antioxidant capacity than previously reported for the same variety [23], although lower than some Criollo and native South American varieties [22, 31-32]. Its ORAC activity was comparable or even higher than that of some commercial cacao from Colombia and Africa [24, 33-34]. These findings support the established correlation between phenolic content and antioxidant activity in cacao [35-36], highlighting the functional potential of the RS extract.

Cytotoxicity effect in macrophages RAW 264.7 and adipocytes 3T3-L: Before phenolic compound testing, the cytotoxicity of the extracts was evaluated. In macrophages, concentrations of 100 and 500 µg/mL of RS increased cell viability, possibly due to the action of epigallocatechins [37]. In 3T3-L1 preadipocytes, a similar effect was observed with RS in the range of 250–1000 µg/mL [19].

Although there are few studies on the cytotoxicity of native varieties, the results are consistent with reports showing no toxic effects at similar concentrations [9, 38]. This suggests that the phenolic composition of Rojo Samuel could confer a good cellular tolerance and safety profile in *in vitro* models.

Anti-inflammatory effect in Macrophages Raw 264.7: RS treatment reduced NO production in a dose-dependent manner, with an IC50 of 221.93 ± 13.37 µg/mL, lower

than CA, possibly due to synergistic phenolic activity [38-39]. This effect may involve NRF2 activation by procyanidin B2, epicatechin, and catechin [40]. However, the IC50 of RS was still higher than values reported for Malaysian cacao powder and cacao shell extracts [9, 38], indicating lower relative potency.

RS and CA did not affect TNFα expression in LPS-stimulated cells, suggesting this pathway was not modulated [9, 37, 41]. Despite the condensed tannin (CT) content indicating phenolic oligomers with biological activity, further studies should assess other pathways (e.g., NF-κB, IL-1β) to clarify the anti-inflammatory potential of native cacao. These findings contrast with studies where cocoa shell extracts or cocoa powder inhibited TNFα [9, 38, 42].

Effects on lipid and triglyceride accumulation in Adipocytes: At a concentration of 150 µg/mL, RS significantly reduced lipid and triglyceride accumulation in adipocytes, unlike previous studies with pure catechin at lower concentrations [43]. This reduction could be attributed to compounds present in the extract, such as gallic acid, chlorogenic acid, rutin, and naringin, known to modulate cellular lipid metabolism [44].

Furthermore, RS also downregulated the expression of PPARγ (81.17%) and FAS (40.20%), suggesting that lipid reduction could be mediated by the inhibition of this key pathway in adipogenesis [45,46]. PPARγ inhibition can directly impact triglyceride synthesis, which is consistent with the observed effects. However, since some phenolic compounds, such as catechin, can activate PPARγ and improve insulin sensitivity [47], the overall impact of RS may depend on the interplay between multiple phenolic compounds in the extract [48].

Scientific Innovation and Practical Implications: This study provides the first comprehensive characterization of the biological activity of phenolic extracts from the native Mexican Criollo Rojo Samuel cacao variety. Unlike previous research on commercial cacaos, this study focuses on a variety- and origin-specific approach.

The results demonstrate the antioxidant, anti-inflammatory, and anti-adipogenic potential of RS extracts, attributed to compounds such as epicatechin, catechin, and sinapic acid, supported by a detailed chemical profile. These findings position Rojo Samuel cacao as a promising source of bioactive compounds for the development of functional ingredients, nutraceuticals, and sustainable products.

CONCLUSIONS

The study, using antioxidant assays, found that Rojo Samuel cacao beans have higher antioxidant activity and a rich profile of phenolic compounds. UHPLC analysis revealed epicatechin and catechin as the main compounds. Extracts from these beans significantly reduce nitric oxide production in RAW 264.7 macrophages, demonstrating antioxidant and anti-inflammatory effects. Furthermore, these extracts influenced the expression of markers linked to cell differentiation and lowered intracellular and triglyceride levels in 3T3-L1 adipocytes, indicating a potential impact on metabolic processes involved in adipogenesis

LIST OF ABBREVIATIONS USED: RS: Rojo Samuel, GAE: Gallic acid equivalents, CA: Catechin equivalents, RE: Rutin equivalents, TE: Trolox equivalents, TFA: Trifluoroacetic acid, DPPH: 1,1-diphenyl-2-picrylhydrazyl; AAPH: 2,2'-azobis(2-methylpropionamidine) dihydrochloride radical; DMEM: Dulbecco's modified Eagle culture medium, FBS: Fetal bovine serum, NCS: Newborn Calf serum, LPS: Lipopolysaccharides, NO: Nitric oxide, TNFα: Tumor necrosis factor alpha, FAS: Fatty acid synthases, PPARγ: Peroxisome proliferator-activated receptors, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, ORO: Oil Red O, PA: Pre-adipocytes (Cells no differentiated), NT: No treatment, ND: No detected.

Competing interests: The authors declare no conflict of interest.

Authors' contributions: L. A.: Drafting, Methodology, Investigation, Analysis, Conceptualization, Review & editing. M.A.V. & D.V.R.: Drafting, Review & editing, Supervision, Methodology, Investigation, Analysis, Conceptualization. L.M.: Review & editing, Supervision, Investigation, Analysis, Conceptualization. M.E.J.F.: Review & editing, Supervision, Investigation, Resources. C.A.A.: Resources, and supervision, and E.L.C.: Review & editing, Resources, Project administration, Funding acquisition, Conceptualization, Supervision, Investigation.

All authors have reviewed and approved the final version of the manuscript for publication.

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