

# Phenolic Compounds from Maté (*Ilex paraguariensis*) Inhibit Adipogenesis in 3T3-L1 Preadipocytes

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Published online: 28 April 2012  
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**Abstract** Leaves of *Ilex paraguariensis* are used to prepare a tea known as maté which is a common beverage in several South American countries. The ethanol extract was fractionated to identify the compounds responsible for the anti-adipogenic activity in 3T3-L1 cells. Extracts of both fresh and dried maté leaves were subjected to column chromatography using molecular permeation to obtain the saponin (20 % yields) and the polyphenol extracts (40 % yields) from the fresh and dried leaves. The phenolic content was determined using high-performance liquid chromatography analysis and the Folin-Ciocalteu method. Also, maté extracts (50 µg/ml to 1,000 µg/ml) did not display cytotoxicity using MTT. The polyphenol extract from the dried leaves was the most effective (50 µg/ml) in the inhibition of triglyceride accumulation in 3T3-L1 adipocytes, and rutin (100 µg/ml) likely accounted for a large portion of this activity. Additionally, maté extracts had a modulatory effect

on the expression of genes related to the adipogenesis as PPAR $\gamma$ 2, leptin, TNF- $\alpha$  and C/EBP $\alpha$ .

**Keywords** *Ilex paraguariensis* · Maté · Adipogenesis · Polyphenols · Saponin · MTT

## Abbreviations

DMEM	Dulbecco's modified Eagle's medium
IBMX	3-isobutyl-1-methylxanthine
TNF $\alpha$	Tumor necrosis factor-alpha
FE	Fresh leaf ethanol extract
DE	Dried leaf ethanol extract

## Introduction

*Ilex paraguariensis* is an important South American crop, known as yerba mate or maté, from which leaves and twigs are used to prepare a tea. Maté is one of the most commonly consumed beverages in several South American countries, including Brazil (especially the southern states), Uruguay, Paraguay, and Argentina. The leaves of *Ilex* species have been extensively studied [1]. In addition to polyphenols such as flavonoids (quercetin and rutin) and phenolic acids (chlorogenic and caffeic acids), maté is rich in caffeine and saponins. The saponins isolated from maté to date are composed of ursolic acid or oleanolic acid as the aglycone and various sugars [1, 2]. Recently, beneficial effects of *I. paraguariensis* have been demonstrated, including antioxidant activity [1–3], a protective effect against induced DNA damage [4] and anti-obesity effects [5, 6].

Obesity is a complex condition involving social, biological and psychosocial factors. A sedentary lifestyle and a high-calorie diet seem to be the most important factors in the

**Electronic supplementary material** The online version of this article (doi:10.1007/s11130-012-0289-x) contains supplementary material, which is available to authorized users.

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development of obesity [7]. Co-morbidities associated with obesity are serious and include both psychosocial and biological factors involving several metabolic problems [8]. Metabolic syndrome is a set of metabolic and hemodynamic abnormalities often present in obese individuals [9]. Adipose tissue is the largest reservoir of energy in the body. This tissue consists of adipocytes that synthesize, store and mobilize fatty acids in response to changes in physiological demands. These processes are highly regulated by genetic, nutritional, hormonal and paracrine mechanisms [10, 11].

Previous studies have shown that the consumption of maté has an anti-obesity and anti-inflammatory effect in animal models [5, 6]. Thus, the aim of the present study was to evaluate the effect on adipogenesis *in vitro* of the main bioactive compounds of maté and to characterize which of these compounds is most likely responsible for the anti-obesity activity.

## Materials and Methods

All chemicals (analytical grade) were purchased from Fluka Chemie (Switzerland) and Merck (Germany). Caffeic acid, chlorogenic acid, gallic acid, rutin and ursolic acid were purchased from Sigma-Aldrich (USA). Matesaponin 4 was isolated as previously described [12].

### Plant Material and Extraction

Leaves from *I. paraguariensis* A. St. Hil. were harvested from a cultivated area, and a voucher specimen (ICN 163413) was deposited at the herbarium of the University. A portion of the material was used fresh, and the residue was dried for 15 days in an air-circulating stove. Hydro-ethanolic extracts were prepared by maceration using fresh (F) or dried (D) leaves (100 g) in 70 % ethanol (1:10, plant: solvent, m/v). After ethanol elimination, the aqueous phases were separately freeze dried to obtain the ethanol extracts from fresh (FE) and dried (DE) leaves. Both ethanol extracts were subjected to column chromatography using molecular permeation (Sephadex LH-20®) with a water:ethanol gradient as an eluent. These fractions were grouped together according to thin-layer chromatography (TLC) using Si gel GF<sub>254</sub> (Sigma-Aldrich®), chloroform:ethanol:acetic acid (100:40:6, v/v) and ethyl acetate:acetone:acetic acid:water (60:20:10:10, v/v) as eluents. The plates were observed under UV<sub>254/366</sub> after being sprayed with anisaldehyde sulfuric acid/100 °C to visualize the saponins or with the Natural Reagent A to visualize the phenolic compounds [13]. The saponin extract (20 % yields) was obtained from the fresh (FS) and dried (DS) leaves, and the polyphenol extract (40 % yields) was obtained from the fresh (FP) and dried (DP) leaves.

### HPLC Analysis of Chlorogenic Acid and Rutin

High-performance liquid chromatography (HPLC) analysis was conducted according to previously described methods [14] using a Shimadzu® Prominence HPLC system (Kyoto, Japan) coupled to an SPD-20A UV/VIS detector. Additionally, an RP-18 column (CLC-ODS (M) 250×4.6 mm i.d., 5-µm particle size) was used coupled to a Waters® pre-column (20 x 3.9 mm i.d., 10-µm particle size). The mobile phase consisted of (A) 2.0 % acetic acid (v/v) and (B) methanol: water (8.5:1.5, v/v). The elution gradient was as follows: 31 % B (0–10 min), 31–56 % B (10–25 min), 56 % B (25–33 min), 56–77 % B (33–45 min), 77–56 % B (45–50 min) and 56–31 % B until 60 min. The flow rate was 0.7 ml/min, and the injection volume was 20 µl. A 340-nm wavelength was used, and the analysis was performed at 23±1 °C. Chlorogenic acid and rutin exhibited retention times of 11.06 and 30.66 min, respectively, when dissolved in methanol:water (50:50, v/v) at 2, 4, 6, 8 or 10 µg/ml. For the chlorogenic acid and rutin calibration curves, the linear equations ( $n=5$ ) were  $y=140094x - 14056$  ( $r^2=0.999$ ) and  $y=81381x+2130.9$  ( $r^2=0.999$ ), respectively. For samples, a solution of each FE and DE sample dissolved in methanol: water (1:1, v/v) was prepared at 0.4 mg/ml. The solutions were filtered through a 0.45-µm membrane (Millipore HVLP), and the data are expressed as means ± SE of triplicate experiments.

### Determination of Phenolic Compounds Using the Folin-Ciocalteu Method

FE and DE samples were tested along with the FS, DS, FP and DP samples. Methanol solutions of gallic acid at 15, 20, 25, 50 or 75 µg/ml were prepared as a standard with a linear equation of  $y=0.0405x-0.4847$  ( $r^2=0.994$ ). Samples of 1 mg/ml were prepared. To conduct the assay, 30 µl of each sample was added to 96-well plates followed by 75 µl of Folin-Ciocalteu reagent (2 N), 30 µl Na<sub>2</sub>CO<sub>3</sub> (15 %) and 15 µl distilled water [15]. After 2 h, the absorbance was read at 750 nm; the data are expressed as gallic acid equivalents (GAE) per g on a dry-weight basis (means ± SE of triplicate experiments) (see [Supplementary Material](#)).

### 3T3-L1 Cell Culture

The 3T3-L1 cell line (ATCC) was cultured to confluence in DMEM medium supplemented with 10 % fetal bovine serum and 10 ml/l penicillin/streptomycin at 37 °C in a 5 % CO<sub>2</sub> atmosphere. Forty-eight hours after achieving confluence (Day 0), the cells were incubated in differentiation medium (DMEM medium supplemented with 10 % fetal bovine serum, 0.25 µM dexamethasone, 10 µg/ml insulin and 0.5 mM IBMX). After 48 h, the cells were exposed to

maturation medium (DMEM medium supplemented with 10 % fetal bovine serum and 5 µg/ml insulin) and cultured for 15 days.

#### Assessment of 3T3-L1 Differentiation

Differentiation was assessed using oil red O staining. Briefly, the cells were incubated with extracts or isolated compounds (50 µg/ml to 100 mg/ml) during the differentiation period. After 15 days, the cells were washed with PBS, fixed in 4 % paraformaldehyde for 30 min and incubated for 1 h with 1 % oil red O solution (Sigma-Aldrich) [16]. After repeated washings with water, the oil red O was dissolved in 100 % isopropyl alcohol, and the optical density was measured on a microplate spectrophotometer at 540 nm.

#### Determination of Cell Viability

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction method described by Mosmann [17] was used, with modifications, to determine cell viability. Briefly, 3T3-L1 cells were used after the period of cell maturation (Day 12), as described above. Extracts were added to the cells at concentrations ranging from 50 µg/ml to 1,000 µg/ml, and reference or isolated compounds were added as follows: ursolic acid at 800, 900 or 1,000 µg/ml; caffeic acid, matesaponin 4 and rutin, all of them at 100, 500 or 1,000 µg/ml. Cells were incubated at 37 °C and 5 % CO<sub>2</sub> for 24 h. Subsequently, wells were washed with 100 µl Hank's buffer solution followed by the addition of 10 µl MTT solution (5 mg/ml). After 3 h (37 °C and 5 % CO<sub>2</sub>), 100 µl of 10 % SDS in 0.01 M HCl was added to lysed the cells, followed by incubation for 18 h (37 °C and 5 % CO<sub>2</sub>). Absorbance was read on a microplate spectrophotometer at 540 nm. The absorbance of control cells (untreated) was considered as 100 % cell viability (see [Supplementary Material](#)).

#### RNA Extraction and cDNA Synthesis

Cell culturing for RNA extraction was performed as described for 3T3-L1 differentiation. For RNA stabilization and protection, all samples were stored in RNAlater (QIAGEN, Valencia, CA, USA) at -80 °C until the time of RNA extraction using an RNeasy<sup>®</sup> tissue kit (QIAGEN) according to the manufacturer's instructions. After the extraction, ~100 µg of RNA was used for cDNA synthesis using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), as described by Xin et al. [18].

#### Quantification of Expression by Real-Time PCR

The expression of the PPARγ2, leptin, TNF-α and C/EBPα genes as well as the constitutively expressed β-actin gene was

analyzed using real-time PCR (see [Supplementary Material](#)). The primers used in this study were designed with the aid of the website <http://fokker.wi.mit.edu/primer3/input.htm>. The real-time PCR was performed using Platinum<sup>®</sup> SYBR GREEN<sup>®</sup> qPCR Supermix UDG (Invitrogen) according to the manufacturer's recommendations. The samples were cycled with a 7300 Real-Time PCR System and analyzed with RQ Study Software (Applied Biosystems). All reactions were performed in triplicate, and the average C<sub>t</sub> value was used to assess gene expression. Relative expression was calculated according to previously described methods [19].

#### Statistical Analysis

Results are expressed as means ± SD; the statistical significance was determined by ANOVA followed by the Dunnett test (\**p*<0.05), (\*\**p*<0.01) and (\*\*\*)*p*<0.001).

## Results and Discussion

### Chemical Composition

Extracts of both fresh and dried maté leaves were made in 70 % ethanol to evaluate the influence of the drying process on the chemical and biological activity of the constituent compounds. Both ethanol extracts were further fractionated to determine the active compounds involved in adipogenesis. Because the main maté constituents are phenolic compounds and saponins, polyphenol and saponin extracts were obtained from fresh and dried maté leaves. HPLC revealed that in the ethanol extracts, the chlorogenic acid content (mg/g on a dry-weight basis) in fresh leaves was twice (10.40±0.04) as high as in dried leaves (5.61±0.02), whereas the rutin content (mg/g on a dry-weight basis) was 6.10±0.21 in fresh leaves and 8.51±0.02 in dried leaves. In dried leaves, Silva et al. [14] found a higher concentration of both chlorogenic acid and rutin in 40 % hydro-ethanolic extract solutions by turbo extraction (2.06 and 0.74 mg/ml, respectively) than by either decoction (1.53 and 0.49 mg/ml, respectively) or infusion (1.75 and 0.55 mg/ml, respectively). Thus, the amount and composition of the chemical compounds obtained may vary according to the type of extraction, solvent and temperature used.

In the phenolic analysis of maté samples, rutin and chlorogenic acid were determined using the Folin-Ciocalteu method, results are expressed as gallic acid equivalents (see [Supplementary Material](#)). The phenolic contents of the ethanol extracts of fresh and dried leaves (151.61 mg and 170.91 mg GAE/g, respectively) were significantly different (*p*<0.05). Of all maté samples, the polyphenol extract of fresh leaves exhibited higher content of total phenols (250.29 mg GAE/g, *p*<0.05), followed by the polyphenol extract and the ethanol extract of dried leaves (180.17 and 170.91 mg GAE/g,

respectively). As expected, the saponin extracts of both fresh and dried leaves exhibited lower contents of phenolic compounds (79.42 and 76.34 mg GAE/g, respectively). Moreover, in relation to the reference compounds, the rutin content was 124.72 mg GAE/g, and the chlorogenic acid content was 179.37 mg GAE/g being this latter note significantly different from that of the polyphenol extract of dried leaves ( $p < 0.05$ ). A previous study [20] reported the phenolic content measured by the Folin-Ciocalteu method in aqueous extracts of *Ardisia compressa*, *I. paraguariensis* and *Camellia sinensis*, demonstrating that *I. paraguariensis* had a higher phenolic content (94.91 mg GAE/g) than *A. compressa* (39.07 mg GAE/g). However, *C. sinensis* had the highest content (148.77 mg GAE/g).

### Adipocyte Differentiation and Gene Expression

Adipogenesis, the differentiation of new fat cells, is considered a dynamic process and is a field of intensive research [11]. Several studies of adipogenesis have been performed using the 3T3-L1 cell line [16, 21–23]. Many phytochemicals are potential anti-obesity agents, and understanding the activities of these compounds during adipogenesis is essential to the development of new treatments for obesity [22, 23]. The differentiation of preadipocytes into adipocytes is associated with an increased number of oil red O-positive cells due to lipid accumulation. We demonstrated that incubation with some *I. paraguariensis* extracts or using isolated compounds reduced the lipid accumulation in a dose-dependent manner ( $p < 0.05$ ) (Fig. 1). This anti-adipogenic effect was achieved at concentrations that did not affect cell viability according to the MTT assay (see [Supplementary Material](#)).

Figure 1 presents data related only to the samples displaying the best inhibition of intracellular lipid accumulation. In this sense, the highest anti-adipogenic effect was detected in the polyphenol extract of dried leaves at 50  $\mu\text{g/ml}$ , followed by the saponin extract of fresh leaves at 100  $\mu\text{g/ml}$  and by the polyphenol extract of fresh leaves at 500  $\mu\text{g/ml}$  ( $p < 0.05$ ). Among the reference substances, rutin exhibited the highest inhibitory activity (100  $\mu\text{g/ml}$ ), followed by chlorogenic acid (300  $\mu\text{g/ml}$ ), matesaponin 4 (500  $\mu\text{g/ml}$ ) and ursolic acid (800  $\mu\text{g/ml}$ ). Caffeic acid did not inhibit intracellular lipid accumulation up to a concentration of 1,000  $\mu\text{g/ml}$  (data not shown). Thus, among the tested samples, phenolic compounds were the most active, and rutin likely accounted for a large portion of the activity detected in the polyphenol extracts. These findings demonstrate that it was possible to obtain a polyphenol-enriched extract exhibiting a potent anti-adipogenic effect.

Adipocyte differentiation depends upon the coordinated regulation of gene expression. Adipogenic transcription factors, including the peroxisome-activated receptor gamma (PPAR $\gamma$ ), the linking-element-binding protein 1c, regulated

by sterols (SREBP-1c) and the proteins that bind to the amplifier CCAAT (CCAAT/enhancer binding proteins, or C/EBPs), play a key role in the complex transcriptional cascade that occurs during adipogenesis [24, 25]. To evaluate the expression of genes related to adipogenesis, we used the samples that inhibited lipid accumulation in 3T3-L1 cells at the lowest concentrations, including the saponin extract from the fresh leaves (FS), polyphenol extracts from the fresh and dried leaves (FP, DP), chlorogenic acid, rutin, ursolic acid and matesaponin 4. In brief, all assayed samples and standards restrained the expression of the PPAR $\gamma$ 2, leptin, TNF- $\alpha$ , and C/EBP $\alpha$  genes, as shown in Table 1 (see also [Supplementary Material](#)).

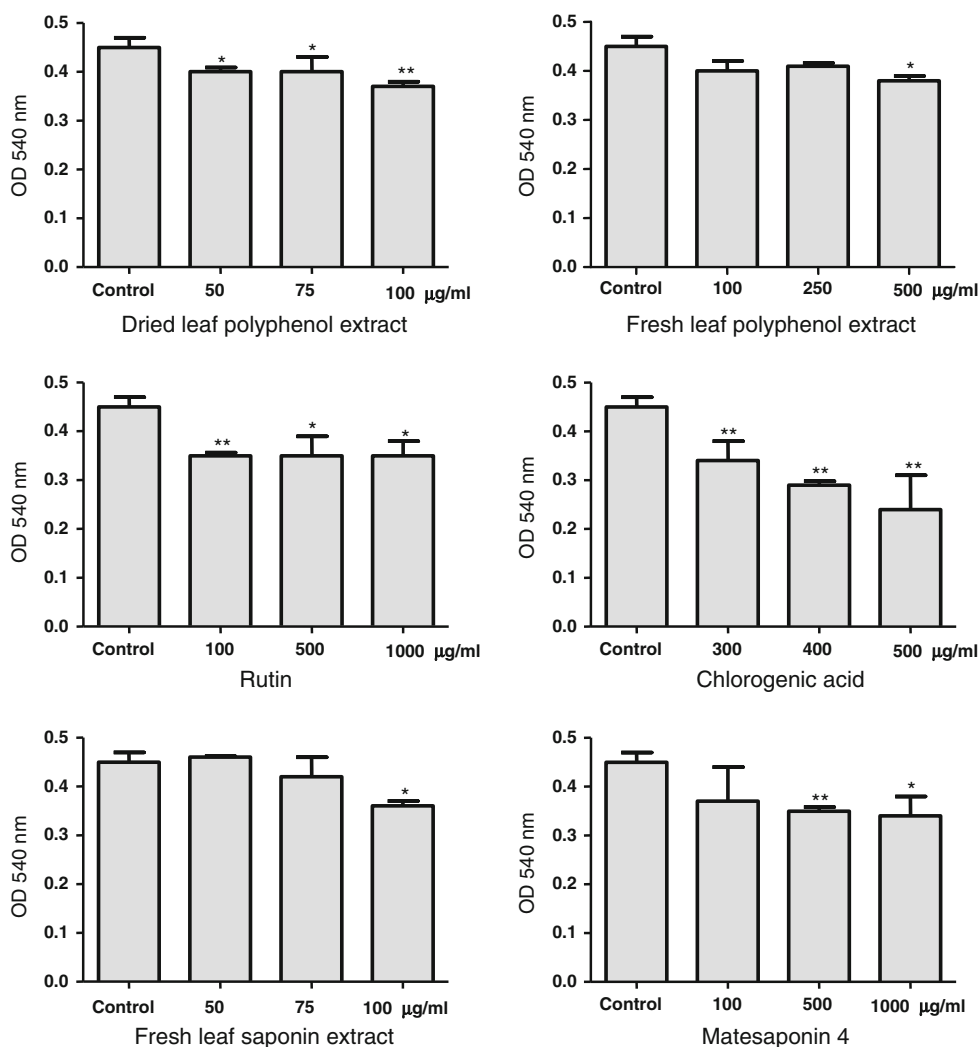
Several studies have shown that PPAR $\gamma$  is the main regulator of adipogenesis and its maintenance is critical to the progression of the final stages of adipocyte differentiation [26]. PPAR $\gamma$ 2 is mainly expressed in adipose tissue and promotes adipocyte differentiation and proliferation, resulting in an increase in adiposity [27]. Several studies have demonstrated that the activation of PPAR $\gamma$  results in the expression of several pro-adipogenic genes, including C/EBP- $\alpha$  [28]. Studies have also demonstrated that the expression of C/EBP- $\alpha$  increases the expression of PPAR $\gamma$  [29]. Thus, the results from the present study are important to the characterization of the anti-adipogenesis mechanism of the bioactive compounds in maté, because these transcription factors are critical to the final stages of adipocyte differentiation and progression. In addition, Hsu and Yen [30] found that rutin exhibits anti-adipogenic activity mediated by the inhibition of the expression of PPAR $\gamma$ 2 and C/EBP $\alpha$ .

Moreover, it is well known that obesity leads to the increased production of several inflammatory cytokines, which play a critical role in obesity-related inflammation and metabolic pathologies. TNF- $\alpha$  is a potent cytokine that induces the production of IL-6, which is the major determinant of the acute phase response [31]. It has been reported that in obese individuals and animal models, the levels of TNF- $\alpha$  and IL-6 are persistently elevated and that a reduction of adipose mass leads to a decrease in the expression levels of these genes. Additionally, TNF- $\alpha$  can modulate leptin secretion by increasing its expression and circulating levels [32, 33]. Our data demonstrated a down-regulation of TNF- $\alpha$  and leptin mRNA expression after the treatment when compared to the control group. In a related finding, Cho et al. [34] showed that supplementation with chlorogenic acid in a diet-induced obesity model in rats enhanced lipid metabolism and the hormones related to obesity, such as leptin.

### Conclusions

To the best of our knowledge, this is the first time that the effects of fractionated *I. paraguariensis* extracts were tested

**Fig. 1** The effects of maté extracts and reference substances on triglyceride accumulation in 3T3-L1 cells expressed as the measurement of absorbance at 540 nm using oil red O assay. \* $p < 0.05$  and \*\* $p < 0.01$  when compared to the control group



on preadipocytes to identify the compounds responsible for the anti-adipogenic activity that was previously demonstrated in crude maté extracts *in vivo*. Our results suggest that the polyphenol extract inhibits the lipid storage in adipocytes, in

part by suppressing the expression of several genes related to adipogenesis. Therefore, an extract enriched in active compounds was obtained having potential to be used in the food and pharmaceutical industries.

**Table 1** Gene expression determined by quantitative real-time PCR to maté extracts and reference substances that inhibited lipid accumulation in 3T3-L1 cells at the lowest concentrations<sup>a</sup>

Sample (µg/ml)	Genes			
	PPAR $\gamma$ 2	C/EBP $\alpha$	Leptin	TNF- $\alpha$
Control	2.36±0.25	4.28±0.45	3.21±0.30	4.03±0.65
FS (100)	1.22±0.12*	2.45±0.32**	1.69±0.45**	2.99±0.42**
FP (500)	1.05±0.09**	2.13±0.23**	1.85±0.42**	3.01±0.53*
DP (50)	1.23±0.13*	1.95±0.29**	1.78±0.38**	3.23±0.44*
CHL (300)	0.97±0.08**	1.99±0.29**	1.81±0.36**	1.98±0.29**
RUT (100)	0.99±0.12**	1.89±0.21**	1.99±0.28**	2.89±0.24*
UA (800)	1.12±0.15**	2.52±0.33**	1.96±0.37**	2.67±0.26*
MS4 (500)	1.00±0.10**	1.96±0.29**	1.87±0.31**	3.13±0.54*

<sup>a</sup> (FS) Saponin of fresh leaf, (FP) Polyphenols of fresh leaf, (DP) Polyphenols of dried leaf, (CHL) Chlorogenic acid, (RUT) Rutin, (UA) Ursolic acid, (MS4) Matesaponin 4. Results represent the average of three independent experiments. \* $p < 0.05$  and \*\* $p < 0.01$  when compared to control group.

**Acknowledgements** We are grateful to the Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for fellowships and financial support.

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