Rock tea (*Jasonia glutinosa* (L.) DC.) polyphenolic extract inhibits triglyceride accumulation in 3T3-L1 adipocyte-like cells and obesity related enzymes in vitro

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*Jasonia glutinosa* (L.) DC., also known in Spain as “té de roca” (rock tea, RT), is an endemic plant species of the Iberian Peninsula and Southern France. Traditionally, it is used in infusions, prepared with the flowering aerial parts, as a digestive and anti-inflammatory herbal tea. Despite the traditional knowledge of this plant as a digestive after meals, there are hardly any scientific studies that support its use. The aim of this study is to assess the effects of RT extract on physiological targets related to metabolic diseases such as obesity. For this purpose, enzyme inhibition bioassays of lipase, α-glucosidase and fatty acid amide hydrolase were carried out in cell-free systems. Similarly, adipocytes derived from 3T3-L1 cells were employed to study the effects of the extract on adipocyte differentiation and triglyceride (TG) accumulation. RT extract was able to inhibit lipase, α-glucosidase and fatty acid amide hydrolase. Furthermore, the extract displayed anti-adipogenic properties in a dose-dependent manner as it significantly reduced TG accumulation during adipocyte differentiation. These results may explain from a molecular perspective the beneficial effects of RT in the prevention of metabolic-associated disorders such as obesity, diabetes and related complications.

1. Introduction

Obesity and overweight are defined as abnormal or excessive fat accumulation that can be harmful to health, being one of the epidemics of this era. According to the World Health Organization, since 1975 obesity has tripled in the world. In 2016, about 40% of adults were overweight, and 13% were obese. This rapid growth has been due to the increase in the consumption of hypercaloric foods and the change in healthy habits, resulting in a positive caloric balance. These are risk factors for non-communicable and chronic diseases such as cardiovascular diseases, diabetes, musculoskeletal disorders and some cancers. Among the healthy habits of the Mediterranean population is the consumption of digestive beverages after meals, traditionally known as teas or infusions. One of these herbal teas is prepared in rural areas of the Iberian Peninsula (Spain) with *Jasonia glutinosa* (L.) DC. (Compositae), popularly known as “té de roca” (rock tea in English, RT). RT is a medicinal plant extended in the Iberian Peninsula and southern France. Traditionally, the infusions and decoctions of the flowering aerial parts of this species have been used for gastrointestinal and digestive problems such as flatulence, diarrhea or abdominal pain. Other reported traditional uses are to treat respiratory diseases, appendicitis, general pain, emesis or depression. Recent studies from our group have shown its spasmylytic effect on duodenal smooth musculature and improvement of gastrointestinal transit. In addition, a beneficial potential in an *in vivo* induced colitis model has been demonstrated, showing anti-inflammatory effects and normalizing intestinal contractility.

The most representative phenolic compounds identified for *J. glutinosa* are hydroxycinnamic acids derived from caffeoylquinic acid and flavonoids such as quercetin glycosides. These bioactive compounds could explain some of the properties attributed to this plant species, including the antioxidant, anti-inflammatory, antihypertensive, antimicrobial activities and those related to the digestive system.
Due to its use as an herbal tea after meals and its phytochemical profile rich in polyphenols, it was hypothesized that RT might exert activities related to metabolic disorders prevention. Polyphenols have been widely studied in the management of obesity, mainly due to its antioxidant and anti-inflammatory properties. For these reasons, the objective of this manuscript was to assess the ability of this extract to scavenge reactive oxygen species and free radicals; the enzymatic activity in targets involved in glucose and fat metabolism, such as α-glucosidase (α-GLU), lipase and fatty acid amide hydrolase (FAAH); and finally, the role of RT in adipogenesis, fat accumulation and TG formation in the 3T3-L1 cell model.

2. Materials and methods

2.1. Reagents and chemicals

For the antioxidant assay, 2,2′-azobis(2-methylpropionamide)-di-hydrochloride (AAPH), 2,7′-dichloro-dihydrofluorescein diacetate (DCFH-DA), 2,4,6-tri(2-pyridyl)s-triazine (TPTZ), ferrous sulfate heptahydrate, iron[iii] sulfate heptahydrate (FeSO4), ferric chloride (FeCl3) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased to Sigma-Aldrich (Madrid, Spain). For enzyme in vitro inhibition assays, α-glucosidase from Saccharomyces cerevisiae, p-nitrophenyl glucopyranoside (pNPG), lipase (type II) from porcine pancreas, p-nitrophenyl butyrate (pNPB), were acquired through Sigma-Aldrich (Madrid, Spain). Fatty acid amid hydrolase (FAAH) inhibitor screening assay kit (Item no 10005196) was obtained by Cayman Chemical Company (Michigan, USA). For cell culture, 3T3-L1 murine pre-adipocytes (CL-173) were obtained by American Type Culture Collection, USA; Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and BCA reagent by Thermo Scientific, Rockford, IL, USA; glycerolphosphate-oxidase kit (NPG), lipase and fatty acid amide hydrolase (FAAH); and finally, the role of RT in adipogenesis, fat accumulation and TG formation in the 3T3-L1 cell model.

2. 2. Preparation of herbal extract

J. glutinosa ethanolic extract (RT extract) was prepared as previously described and a plant voucher is kept at Universidad San Jorge herbarium (ref. 001-2012). Briefly, 40 g of dried ground plant material was extracted using ethanol as solvent in a Soxhlet apparatus for 4 h. The solvent was then removed using a rotavapor and the extract was kept at −20 °C. The phytochemical composition of the extract (Table 1), which was previously reported, reveals a high proportion of phenolic compounds.6

2.3. Antioxidant assays

2.3.1. Oxygen radical antioxidant capacity (ORAC) assay.

The antioxidant capacity of RT was determined by scavenging peroxyl radicals using a fluorometric probe. A range of selected concentrations of Trolox (0.2–1.6 μM) was prepared. Different concentrations of RT were dissolved in 0.075 M—PBS buffer, pH 7.4 and placed into the wells. RT and Trolox were incubated with 120 μL of fluorescein (116 nM) in 96-well plates for 10 min at 37 °C. Then, 60 μL of AAPH (0.04 M) was added and fluorescence was measured for 93 min at 485 nm (excitation) and 520 nm (emission), in a Synergy H1 Hybrid Multi-Mode Reader (Biotek, Bad Friedrichshall, Germany). Results were expressed as μmol Trolox equivalents (TE) per mg RT.

2.3.2. Ferric reducing antioxidant power (FRAP) assay. RT was also tested through the FRAP assay. FeSO4 (6.25–200 μM) was used as standard compound and results were expressed as μmol Fe2+ per g sample. RT was dissolved in MeOH. FeSO4 and RT were mixed with FRAP reagent (10 mM TPTZ; 20 mM FeCl3; acetate buffer pH 3.6). RT and FeSO4 absorbance were measured in 96-well plate for 10 min at 37 °C at 595 nm in a Synergy H1 Hybrid Multi-Mode Reader.

2.4. Enzyme inhibition assays

2.4.1. α-Glucosidase (α-GLU) inhibition. The inhibition of α-GLU was evaluated using a 96-microplate reader based on the previous method. Each well contained 100 μL α-GLU 1 U ml−1 and 50 μL of the different concentrations of RT extract or reference compound. After preincubation for 10 min, 50 μL of 3.0 mM pNP (solved in phosphate buffer 20 mM, pH 6.9) was added to start the reaction, incubated at 37 °C for 20 min and stopped by adding 2 ml of 0.1 M Na2CO3. Controls wells contained buffer in place of the sample, and blanks buffer instead of the enzyme. The absorbance was measured at 405 nm. The results on α-GLU inhibition were expressed as percentage of enzyme inhibition, using the eqn (1)

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\text{Inhibition (%) = } \left[ \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right] \times 100.\]

2.4.2. Lipase inhibition. The method for measuring lipase inhibition was based on a previous protocol. Each well contained a mixture of 40 μL of sample and 40 μL of reference compound. After preincubation for 10 min, 50 μL of 3.0 mM pNP (solved in phosphate buffer 20 mM, pH 6.9) was added to start the reaction, incubated at 37 °C for 20 min and stopped by adding 2 ml of 0.1 M Na2CO3. Controls wells contained buffer in place of the sample, and blanks buffer instead of the enzyme. The absorbance was measured at 405 nm. The results on lipase inhibition were expressed as percentage of enzyme inhibition, using the eqn (1)
lipase type II (2.5 mg ml$^{-1}$ prepared in Tris-Buffer pH 7.0), and pre-incubated 15 min. Then, 20 µl of pNPB (10 mM) was added to each well and incubated for another 15 min at 37 °C. Controls wells contained buffer in place of the sample, and blanks buffer instead of the enzyme. Finally, absorbance was read at 405 nm. Orlistat was used as reference inhibitor. The inhibitory activity was calculated using the eqn (1).

2.4.3. Fatty acid amide hydrolase (FAAH) inhibition. The in vitro direct inhibition of the FAAH enzyme was assessed with a commercial kit and following manufacturer's instructions (Cayman, item no. 10005196). JZL 195, included in the kit, was used as reference inhibitor. Fluorescence was measured using an excitation wavelength of 340–360 nm, and 450–465 nm for emission wavelength.

2.5. 3T3-L1 cell culture

3T3-L1 murine pre-adipocytes were grown in DMEM with 10% FBS as previous described.16 Two days after confluence, cells were differentiated into adipocytes with 10% FBS/DMEM, insulin (1.67 µM), dexamethasone (1 µM), and isobutylmethylxanthine (500 µM) for two days, and then with 10% FBS/DMEM supplemented only with insulin for two more days. Subsequently, cells were cultured with 10% FBS/DMEM, adding fresh medium every other day. Cells were maintained at 37 °C in a humidified 5% CO$_2$ atmosphere. Treatment was made introducing RT extract at different concentrations into differentiation days. RT extract was dissolved in the different culture mediums. Conditions without the extract were always tested for control values. Previously, different concentrations of sample were tested in 3T3-L1 pre-adipocytes by the MTT in order to avoid cytotoxic doses. After treatment, cells were thoroughly washed with PBS and frozen to −80 °C.

2.5.1. Triglycerides (TG) quantification. TG content was measured photometrically in cell lysates using a glycolphosphosphate-oxidase method (#OSR60118) according manufacturer's instructions. The results were normalized with proteins concentration. For protein determination, cells were lysed in 0.3 N NaOH, 0.1% SDS and measurements were performed using BCA reagent.

2.5.2. Oil red O. Oil red O stain in 3T3-L1 cells was performed as described previously.17 The oil red O dye was added to the cell culture and incubated for 15 minutes with stirring and subsequently removed. The dye was dissolved with isopropanol to subsequently quantify by spectrophotometry at 500 nm.

2.6. Statistical analysis

Results are presented as mean ± standard error of the mean of experiments performed at least in triplicates. IC$_{50}$ values were calculated by nonlinear regression and statistical analysis was performed using Graph Pad Prism version 6.

3. Results

3.1. RT extract exerts in vitro antiradical properties

The in vitro antioxidant activity of RT extract was evaluated using two indicative anti-radical assays; ORAC and FRAP values were 2.72 µmol TE per mg and 52.09 µmol Fe$^{2+}$ per g of RT, respectively.

3.2. RT extract inhibits α-glucosidase, lipase and fatty acid amide hydrolase

RT extract was evaluated with different important physiological enzymes. The extract was able to inhibit α-GLU and lipase (Fig. 1A and B). For both enzymes, the response was in a dose dependent manner. For α-GLU inhibition, the extract had a similar profile as acarbose. For lipase, the maximum inhibition observed was 60% at the highest concentration. The extract also showed capability to reduce FAAH activity, but high inhibitions were only reached at approximately 1 mg ml$^{-1}$ (Fig. 1C). This inhibition was also dose dependent.

RT extract IC$_{50}$ values of all the enzymatic assays differed considerably from those of the reference inhibitors (Table 2). For the inhibition of α-GLU, IC$_{50}$ was the closest to reference inhibitor, being 2052.32 and 378.92 µg ml$^{-1}$ for the extract and acarbose, respectively. However, it must be considered that a crude herbal extract was assayed without further purification.

3.3. RT extract inhibits adipogenesis and triglyceride accumulation in 3T3-L1 adipocyte-like cells

The MTT assay in preadipocytes treated with RT extract revealed that it was not cytotoxic in the range 0–200 µg ml$^{-1}$. However, for subsequent assays, authors decided to test doses not exceeding 100 µg ml$^{-1}$ in order to minimize toxicity during cell differentiation and reproduce physiological concentrations. 3T3-L1 cells became enlarged adipocytes after treatment with differentiation medium. However, supplementation of this medium with different concentrations of RT extract produced a decrease in adipogenesis, decreasing the number of mature adipocytes and remaining pre-adipocytes without differentiation, observing these effects in a dose-dependent fashion (Fig. 2).

This decrease in adipogenesis was confirmed after the analysis of the amount of fat accumulated in adipocytes. The amount of fat deposits, stained by oil red O, decreased when the cells were treated with RT extract as can be seen in microscope images (Fig. 2). Mature adipocytes were able to form fat deposits, but the number of adipocytes and lipid droplets was lower as the extract dose increased.

In addition, quantification of the lipophilic dye confirmed the decrease in fat accumulation during the adipocyte maturation in a dose-dependent and significant manner for both tested concentrations (Fig. 2), corroborating the observed effects under the microscope.

Moreover, according to the adipogenesis reduction, a delipidating effect was observed when cells were treated with RT extract. TG accumulation was significantly decreased for all
concentrations tested of the extract (Fig. 3A). At 100 µg ml$^{-1}$, the TG accumulation was reduced to half, observing the highest effect, without inducing cytotoxicity (Fig. 3B).

4. Discussion

Recent studies validated the traditional use of RT as a digestive herbal tea.\textsuperscript{5,6} Its phytochemical profile has also been recently characterized, highlighting the presence of phenolic acids (such as derivatives of caffeoylquinic acid), flavonoids (as derivatives of quercetin) and some pigments (such as lutein).\textsuperscript{6,7} This composition allows RT extract to exert excellent antioxidant properties, assessed by different authors (superoxide radical scavenging, DPPH and ABTS$^+$ methods),\textsuperscript{6,7} but to the best of authors knowledge, it is the first time that ORAC and FRAP values are reported for this polyphenolic extract which translates into a global profile of the antioxidant capacity of \textit{J. glutinosa}. Other plants with a high content in caffeoylquinic acids such as \textit{Artemisia frigida}, \textit{Cynara scolymus}, \textit{Sargentodoxa cuneata}, \textit{Solanum betaceum} or \textit{Persea americana} were analyzed to determine their ORAC capacity showing 2.91 µmol TE per ml, and 0.4, 0.015, 0.2–0.3 and 0.47 µmol TE per mg, respectively,\textsuperscript{18–22} RT extract reached 2.72 µmol TE per mg. In previous FRAP studies, fourteen methanol extracts of \textit{Smallanthus sonchifolius} (high content in caffeoylquinic acids) showed 31–66 TE per g of sample for FRAP assay\textsuperscript{23} and two different concentrations (2.5 µM and 5 µM) of 5-caffeoylquinic acid (5-CQA) exerted 72.53 and 114.22 µmol Fe$^{2+}$ per g, respectively.\textsuperscript{24} On the other hand, RT extract reached 52.09 µmol Fe$^{2+}$ per g. Considering these results, the antioxidant activity of RT is comparable to other herbal teas and/or greater than other plants with caffeoylquinic acids. These data are important as ORAC and FRAP values are used by different health authorities to compare the antioxidant power of food or beverages. It has

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\textbf{Enzyme} & \textbf{RT extract} & \textbf{Reference inhibitor} & \textbf{Student’s t-test} \tabularnewline
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\textit{α}-GLU & 2052.32 ± 895.14 & 378.92 ± 182.51 (Acarbose) & $p = 0.055$ \tabularnewline
Lipase & 891.50 ± 65.4 & 0.74 ± 0.51 (Orlistat) & $p = 0.002$ \tabularnewline
FAAH & 118.39 ± 98.11 & 0.02 ± 0.009 (JZL 195) & $p = 0.174$ \tabularnewline
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\caption{Comparative of different IC$_{50}$ of \textit{in vitro} enzyme inhibition assays. IC$_{50}$ were calculated with nonlinear regression.}
\end{table}

Student’s $t$-test was performed comparing the RT extract with the reference, being: ns, not significant; and **, $p < 0.01$.

Fig. 1 Activity of RT extract on \textit{in vitro} enzymatic inhibition assays. IC$_{50}$ were calculated by non-linear regression. All concentrations were tested at least in triplicate, and each point represents mean ± SEM. (A) \textit{α}-GLU inhibition performed by RT extract and acarbose as drug reference inhibitor. (B) Lipase inhibition by RT extract and orlistat as drug reference inhibitor. (C) FAAH inhibition by RT extract and JZL 195 as reference inhibitor.
been widely studied that oxidative stress play an important role in the pathogenesis of many diseases, including those related to metabolic syndrome, such as obesity and type 2 diabetes. In fact, ROS overproduction may lead to obesity-induced inflammation Therefore, the antioxidant capacity of this plant and the ability to scavenge superoxide radical, showed in previous studies, could help to prevent several metabolic disorders.

Previous studies have shown that extracts rich in polyphenols have a great capacity to inhibit enzymes involved in glucose and fat metabolism, such as α-GLU and lipase. This study demonstrates, for the first time, the RT extract ability to inhibit these enzymes. However, there are studies that show similar properties with other extracts where RT extract compounds are present. For instance, chlorogenic acids are widely distributed in other plant species, including food and beverages. These have been extensively studied due to their possible beneficial effects on the metabolic syndrome pathologies. A study has demonstrated the ability of plants from Asteraceae (=Compositae) family, with a high content of caffeoylquinic acids, to inhibit the activity of digestive enzymes (α-amylase, α-, β-GLU and lipase) related to type II diabetes and obesity. In addition, derivatives of caffeoylquinic acid from Artemisia princeps leaves were identified as natural inhibitors of the tyrosine phosphatase 1B protein, a potential therapy for diabetes, obesity and cancer. But RT polyphenolic extract not only contains caffeoylquinic acid derivatives, but other phytochemicals previously described with therapeutic potential, such as flavonoids or pigments. Begonia nelumbifolia methanolic extract, containing organic acids, carotenoids and flavonoids (oxalic acid, ascorbic acid, lutein, β-carotene, quercetin and rutin), presented antioxidant and anti-α-GLU activity. In addition, in vitro and in silico studies have shown that quercetin-derived flavonoids have the capacity to inhibit enzymes such as α-GLU and lipase. Carotenoid-rich extract (including lutein and β-carotene among others) from Oedogonium intermedium algae has shown inhibitory effects against several metabolically important enzymes, including α-amylase, α-GLU, lipase and hyaluronidase. This is also the first time that RT extract shows ability to inhibit FAAH. Pharmacological inhibition of this enzyme could prevent leptin-mediated effects on body weight and food intake. Furthermore, endocannabinoid signaling regulates metabolic processes and feeding, linked to the development of obesity. So far there is no study that relates caffeoylquinic acids or pigments presented in RT with a possible FAAH inhibition. The inhibition of this enzyme could explain the anti-inflammatory and analgesic effects observed in previous studies with this

![Figure 2](https://example.com/fig2.png)

**Fig. 2** Percentage of oil red O and microscope images (×40) of 3T3-L1 cell cultures with oil red O staining, treated with different RT extract concentrations during differentiation process. Each column in the figure represents the mean ± SEM of the quadrupled results from independent experiments, and significant differences to control (0 µg ml⁻¹) were calculated by one-way ANOVA (Dunnett’s multiple comparisons test). ** (p < 0.01), *** (p < 0.001).
Another approach of this study was to demonstrate the effect of RT in relation with adipocytes. The decrease in adipogenesis was also demonstrated for the first time, in addition to a delipidating effect. However, there are other studies with flavonoids and caffeoylquinic acids, or extracts rich in them, that could be consistent with the effects observed in 3T3-L1 cells. In this cell line, the impact of coffee fruit on adipogenesis has been tested by measuring oil red O staining and the amount of glycerol released. These fruits are rich in chlorogenic acid, caffeine and caffeic acid, and showed antiadipogenic activity and increased glycerol release. In vivo studies also suggest the potential applications of polyphenols for obesity. For instance, dicaffeoylquinic acids from “Kuding tea” leaves (Ilex kudingcha) have shown potential against obesity in high-fat diet fed (HFD) mice, acting on fat accumulation and intestinal microbiota. Also, decaffeinated green coffee extract showed lower insulin resistance and downregulated genes involved in adipogenesis and inflammation in HFD mice. Another study with HFD rats supplemented with isolated 5-caffeoylquinic acid (5-CQA) improved metabolism disorders, regulating the gene expression of PPARα and LXRα, involved in multiple intracellular signaling pathways. In this line, another study showed that HFD rats supplemented with 5-CQA reduced obesity, macrophage infiltration and steatosis, decreasing the expression of NF-κB and inflammatory cytokines, but increasing the expression of PPARγ. However, there are no in vivo studies yet with RT extract in obese animals.

All these studies with phytochemicals that are also present in RT could explain why this extract could have potential benefits in the treatment or prevention of disorders associated with the metabolic syndrome. However, these compounds do not act isolated in the extract, so there might exist synergies and mechanisms that should be deeply studied. Moreover, there are few randomized clinical trials performed with chlorogenic acids or quercetin that suggest protective properties in metabolic disorders.

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Conflicts of interest
The authors declare no conflict of interest.

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