Pomegranate polyphenols and urolithin A inhibit α-glucosidase, dipeptidyl peptidase-4, lipase, triglyceride accumulation and adipogenesis related genes in 3T3-L1 adipocyte-like cells

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Abstract

Ethnopharmacological relevance: pomegranate fruit is considered an antidiabetic medicine in certain systems of traditional medicine. In addition, pomegranate polyphenols are known as powerful antioxidants with beneficial effects such as the reduction of oxidative / inflammatory stress and the increase of protective signalling such as antioxidant enzymes, neurotrophic factors and cytoprotective proteins.

Aim of the study: this work evaluates the effects of pomegranate juice, its main polyphenols known as ellagic acid and punicalagin, as well as its main metabolite urolithin A, on physiological and pharmacological targets of metabolic diseases such as obesity and diabetes.

Materials and methods: for this purpose, enzyme inhibition bioassays of lipase, α-glucosidase and dipeptidyl peptidase-4 were carried out in cell-free systems. Similarly, adipocytes derived from 3T3-L1 cells were employed to study the effects of ellagic acid, punicalagin and urolithin A on adipocyte differentiation and triglyceride (TG) accumulation.

Results: pomegranate juice, ellagic acid, punicalagin and urolithin A were able to inhibit lipase, α-glucosidase and dipeptidyl peptidase-4. Furthermore, all tested compounds but significantly the metabolite urolithin A displayed anti-adipogenic properties in a dose-dependent manner as they significantly reduced TG accumulation and gene expression related to adipocyte formation such as adiponectin, PPARγ, GLUT4, and FABP4 in 3T3-L1 adipocytes.

Conclusion: these results may explain from a molecular perspective the beneficial effects and traditional use of pomegranate in the prevention of metabolic-associated disorders such as obesity, diabetes and related complications.

Key words: polyphenols; urolithin A; lipase; α-glucosidase; dipeptidyl peptidase-4; 3T3-L1.

Abbreviations

α-glucosidase (α-GLU), dipeptidyl peptidase-4 (DPP-4), peroxisome proliferator-activated receptor gamma (PPARγ), glucose transporter type 4 (GLUT4), fatty acid binding protein 4 (FABP4).

List of compounds studied

ellagic acid (CAS: 476-66-4), punicalagin (CAS: 65995-63-3), urolithin A (CAS: 1143-70-0).

1. Introduction

Pomegranate (Punica *granatum* L.) has traditionally been used for the prevention and treatment of many disorders but particularly in Unani medicine for diseases in relation with the metabolism of carbohydrates and lipids (Ismail et al., 2012; Xu et al., 2009). The interest in this fruit as a nutritional or medicinal product and its therapeutic applications have significantly increased in recent years due to its possible beneficial effects on health, based on the presence of polyphenols, which can protect the human body from free radicals, oxidative processes and the progression of many chronic diseases. Studies on pomegranate juice composition show that the main components are polyphenols, highlighting the presence of punicalagin, ellagic acid, flavonoids and anthocyanins, among others (Les et al., 2017, 2015). Many of the beneficial effects alleged on the consumption of pomegranate juice (PJ) are currently explained by the antioxidant properties of its components. Among such beneficial effects are the cardioprotective, neuroprotective and beneficial properties on metabolic disorders. Ellagitannins are hydrolysable tannins present not only in pomegranate, but also found in walnuts, berries and aged wines. These, once consumed, are hydrolyzed in the intestine by releasing ellagic acid, which is processed by the intestinal microflora into urolithins (García-Niño and Zazueta, 2015; Tang et al., 2017).

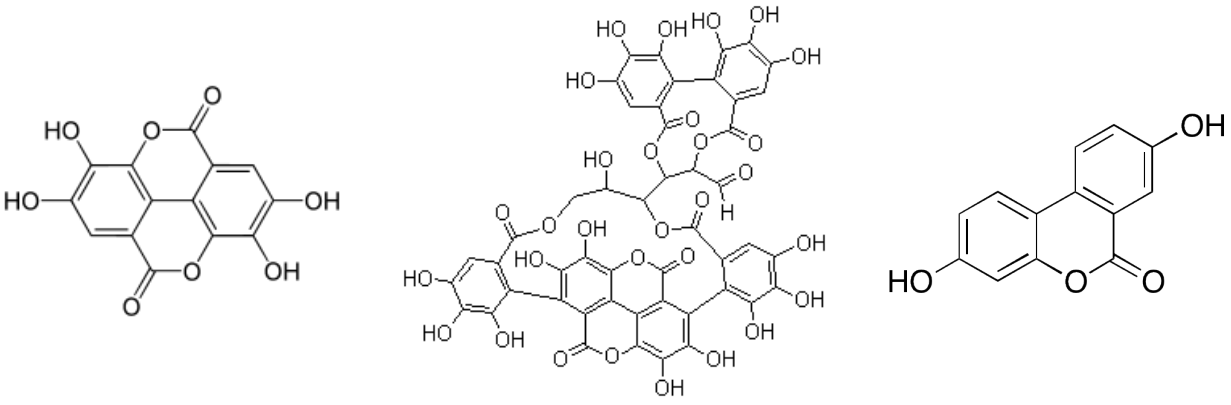
Urolithins are gaining attention in recent years and studies have shown that these metabolites, generated by the intestinal microbiota, exert antiproliferative effects in cancer cells and anti-inflammatory activities improving the endothelial function (Spigoni et al., 2016). In addition, it has recently been reported that urolithins ameliorate life span of worms as well as muscle strength in rodents (Ryu et al., 2016) and triglycerides accumulation in human cultures of adipocytes and hepatocytes (Kang et al., 2016). However, the effects of urolithin A in the expression of genes related to adipocyte differentiation has not been clearly established yet.

Polyphenols have been widely studied due to their antioxidant and anti-inflammatory potential and their role in preventing several diseases such as hypertension, diabetes, cancer and neurodegenerative diseases (Manach et al., 2004; Michalska et al., 2010).

Recently, the role of polyphenols in obesity, an increasingly serious health problem of the population (WHO, 2017), has been demonstrated through clinical trials (Farhat et al., 2017). It has been suggested that various polyphenols can exert beneficial effects by inhibiting adipocytes differentiation, decreasing fatty acids

synthesis, increasing energy expenditure or inhibiting digestive enzymes (Gu et al., 2011; Matsui et al., 2005; Min et al., 2013; Stohs and Badmaev, 2016). In this sense, it is important to study the bioactive properties of isolated polyphenols in order to understand the pharmacological properties of medicinal plants and extracts used in metabolic diseases.

The objective of this study was to evaluate if pomegranate juice and its main polyphenols (ellagic acid, punicalagin and the gastrointestinal metabolite urolithin A (Figure 1)) inhibit pharmacological targets of obesity and diabetes such as lipase, α-glucosidase and dipeptidyl peptidase-4, as well as triglyceride formation and gene expression in the 3T3-L1 cell line. Lipase and α-glucosidase are involved in the hydrolysis of triglycerides and complex carbohydrates respectively in the gastrointestinal tract, whereas dipeptidyl peptidase-4 is in relation with incretin levels, playing also a major role in glucose metabolism (Bessesen and Van Gaal, 2017). The adiponectin, PPARγ, GLUT4 and FABP4 genes are important regulators in adipose tissue and in the metabolism of glucose and fatty acids (Irudayaraj et al., 2016; Poulsen et al., 2012). The hypothesis is that pomegranate polyphenols might exert interesting bioactive properties through the interactions with the enzymes and genes selected for this study. Gene expression has been used as a marker of adipocyte differentiation. The enzymes are current targets in the pharmacotherapy of obesity (lipase) and type 2 diabetes (α-glucosidase and dipeptidyl peptidase-4).



Ellagic acid Punicalagin Urolithin A

Figure 1. Structure of ellagic acid, punicalagin and the main gastrointestinal metabolite (urolithin A).

2. Materials and methods

2.1. Reagents and chemicals:

Pomegranate pure juice (Rabenhorst®) was acquired from a specialized shop (expiration date and batch: 04.03.2016; 11:57), lyophilized using a VIRTIS Genesis 25EL lyophilizer and analysed phytochemically previously by our group (Les et al., 2017, 2015). α-glucosidase, p-nitrophenyl glucopyranoside (pNPG), lipase type II, p-Nitrophenyl butyrate (pNPB), punicalagin and ellagic acid were acquired through Sigma-Aldrich (Madrid, Spain). Urolithin A was purchased from Scionix (Canada). Dipeptidyl peptidase-4 Inhibitor Screening Assay Kit (Item nº 700210) was obtained by Cayman Chemical Company (Michigan, USA).

2.2. Enzyme inhibition assays:

Lipase, α-glucosidase (α-GLU) and dipeptidyl peptidase-4 (DPP-4) were selected because they are pharmacological targets for the treatment of obesity and diabetes and their inhibitors are currently approved as anti-obesity and antidiabetic drugs.

2.2.1. α-GLU inhibition:

The inhibition of α-GLU was evaluated using a 96-microplate reader based on the method described by Kim et al. using α-glucosidase from *Saccharomyces cerevisiae* (Kim et al., 2005). Each well contained 100 µL α-GLU (1.0 U/mL) and with 50 µL of the different concentrations of the tested or reference compounds. After preincubation for 10 min, 50 µL of 3.0 mM pNPG (dissolved 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 Na_2CO_3. The absorbance was measured at 405 nm. The results of the juice on the α-GLU inhibition were expressed as percentage of enzyme inhibition (Equation 1).

$$\text{Equation 1: Inhibition (\%)} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100.$$

2.2.2. Lipase inhibition:

The method for measuring lipase inhibition was based on a previous protocol with some modifications using a 96-microplate reader (Spínola et al., 2017). Each well contained 40 µL of tested sample and 40 µL of lipase type II from porcine pancreas (2.5 mg/mL prepared in Tris-Buffer (100mMTris–HCl and 5mM CaCl2, pH 7.0)). After preincubation of 15 min, 20 µL of 10mM pNPB solution was added to each well. After

121 incubation of another 15 min at 37ºC, absorbance was read at 405 nm. Orlistat was used as positive inhibitor.
122 The inhibitory activity was calculated using the equation 1.
123
124 **2.2.3. DPP-4 inhibition:**
125 DPP-4 inhibition was measured using the fluorogenic substrate Gly-Pro-Aminomethylcoumarin (AMC) with
126 a commercial kit (Cayman, item no. 700210). The authors followed the kit instructions, reading the
127 fluorescence using an excitation wavelength of 350-360 nm and an emission of 450-465 nm. PJ and urolithin
128 was tested at four different concentrations whereas punicalagin and ellagic acid were tested at three
129 concentrations. Sitagliptin was used as positive control and reference inhibitor of the enzyme. The
130 percentage of inhibition of the PJ and the other compounds were determined with the equation 2.
131 Equation 2: Inhibition (%) = [(Initial Activity - Inhibitor) / Initial Activity] x 100.
132
133 **2.3. 3T3-L1 cell culture:**
134 3T3-L1 murine pre-adipocytes (CL-173; American Type Culture Collection, USA) were grown in
135 Dulbecco's Modified Eagle Medium (DMEM) with 10 % fetal bovine serum (FBS, both from Thermo
136 Scientific, Rockford, IL, USA) as previous described (Gamundi-Segura et al., 2015). Two days after
137 confluence, cells were differentiated into adipocytes with 10% FBS/DMEM, 1.67 µM insulin (Actrapid,
138 Novo Nordisk, Denmark), 1 µM dexametasone (D4902, Sigma-Aldrich), and 500 µM
139 isobutylmethylxanthine (I5879, Sigma-Aldrich) for two days, and then with DMEM 10%v/v FBS
140 supplemented with 1.67 µM insulin for two more days. Subsequently, cells were cultured with 10%
141 FBS/DMEM, adding fresh medium every other day. Cells were maintained at 37 °C in a humidified 5 % CO_2
142 atmosphere. The experiments were carried out in 12-wells plates. The PJ and its main polyphenols were
143 introduced at different concentrations into differentiation medium and insulin medium. Previously, different
144 concentrations of samples were tested in order to avoid toxics doses. For the isolated polyphenols, 0.5% v/v
145 of DMSO was used to dissolve them. After treatment, cells were thoroughly washed with PBS and frozen to
146 -80ºC.
147
148 **2.4. Triglycerides**

TG content was measured photometrically in cell lysates using a glycerolphosphate-oxidase method (#OSR60118, Beckman Coulter, Fullerton, CA, USA) according manufacturer's instructions. For protein determination, cells were lysed in 0.3 N NaOH, 0.1 % SDS and measurements were performed using BCA reagent (Thermo Scientific).

2.5. Gene expression

RNA was isolated using TRizol Reagent and treated with DNAse using commercial kits following manufacturer's instructions. (Thermo Scientific). RNA was subsequently retrotranscripted (Applied Biosystems) to cDNA using the High capacity cDNA retrotranscription kit (Thermo Scientific). The expression of the targeted genes was measured with SYBRgreen I (Thermo Scientific) in a StepOnePlus thermocyler (Thermo Scientific). Gene expression was calculated by the -ddCT method (Livak and Schmittgen, 2001) and expressed as the relative change to either actin or AFABP.

2.6. Statistical analysis

Results are presented as mean ± standard error of experiments performed 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. Enzyme inhibition assays:

PJ exhibited an inhibition of α-GLU with a similar profile to acarbose, a reference inhibitor of this enzyme (Figure 2). Its main polyphenols and the metabolite urolithin A also inhibited this enzyme, being more potent than PJ and acarbose. IC_{50} values were 0.0055, 0.015, 0.025, 0.38 and 1.01 mg/mL for punicalagin, urolithin-A, ellagic acid, acarbose and PJ respectively, being punicalagin and urolithin A the best inhibitors.

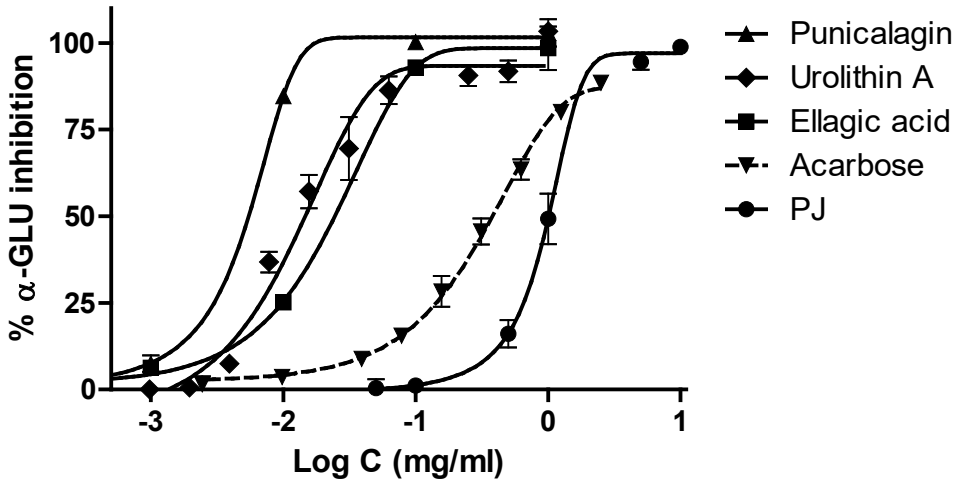


Figure 2. α-glucosidase inhibition performed by pomegranate polyphenols, PJ and acarbose.

Lipase was also inhibited in a dose-response manner (Figure 3). All tested samples were less potent than orlistat but they resulted more efficient than the reference inhibitor. PJ was only able to inhibit lipase at very high concentrations. IC_{50} values were 0.00074, 0.032, 0.092, 0.16 and 2.50 mg/mL for orlistat, urolithin-A, ellagic acid, punicalagin and PJ respectively.

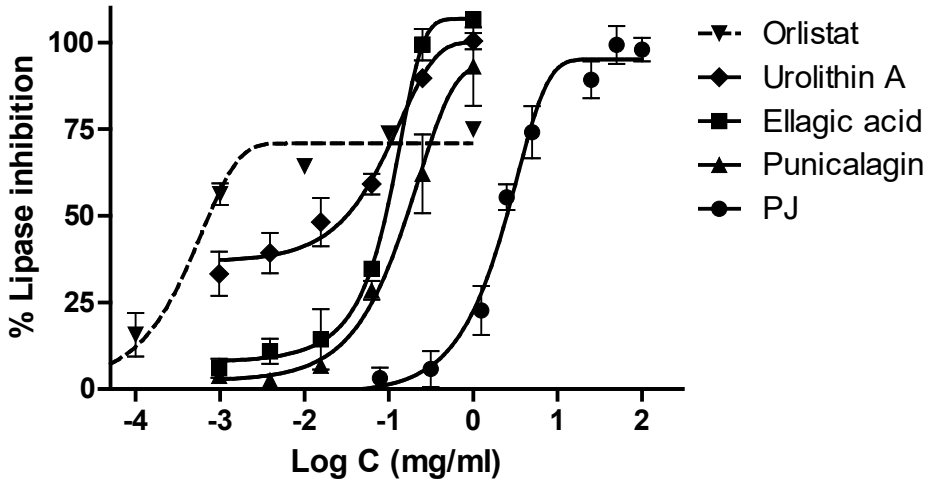


Figure 3. Lipase inhibition by pomegranate polyphenols, PJ and orlistat.

In the DPP-4 inhibition bioassay, the difference observed between sitagliptin, an antidiabetic oral drug, and PJ and its compounds was much more clear (Figure 4). PJ was able to inhibit the enzyme only at high doses with an IC_{50} value of 0.96 mg/mL. Pomegranate polyphenols were better DPP-4 inhibitors, with an IC_{50} 0.025, 0.059 and 0.095 mg/mL for ellagic acid, punicalagin and urolithin-A, respectively. IC_{50} value of sitagliptin, selective inhibitor, was $9.14 \cdot 10^{-5}$ mg/mL.

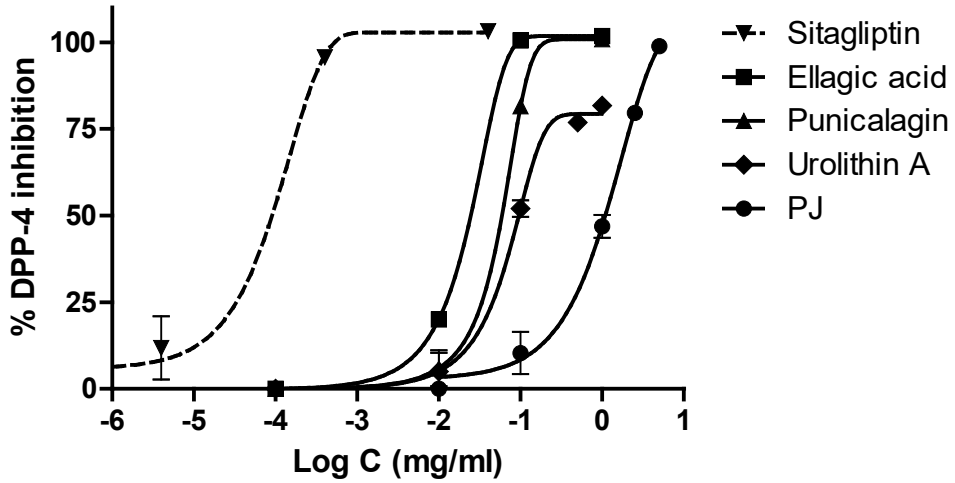
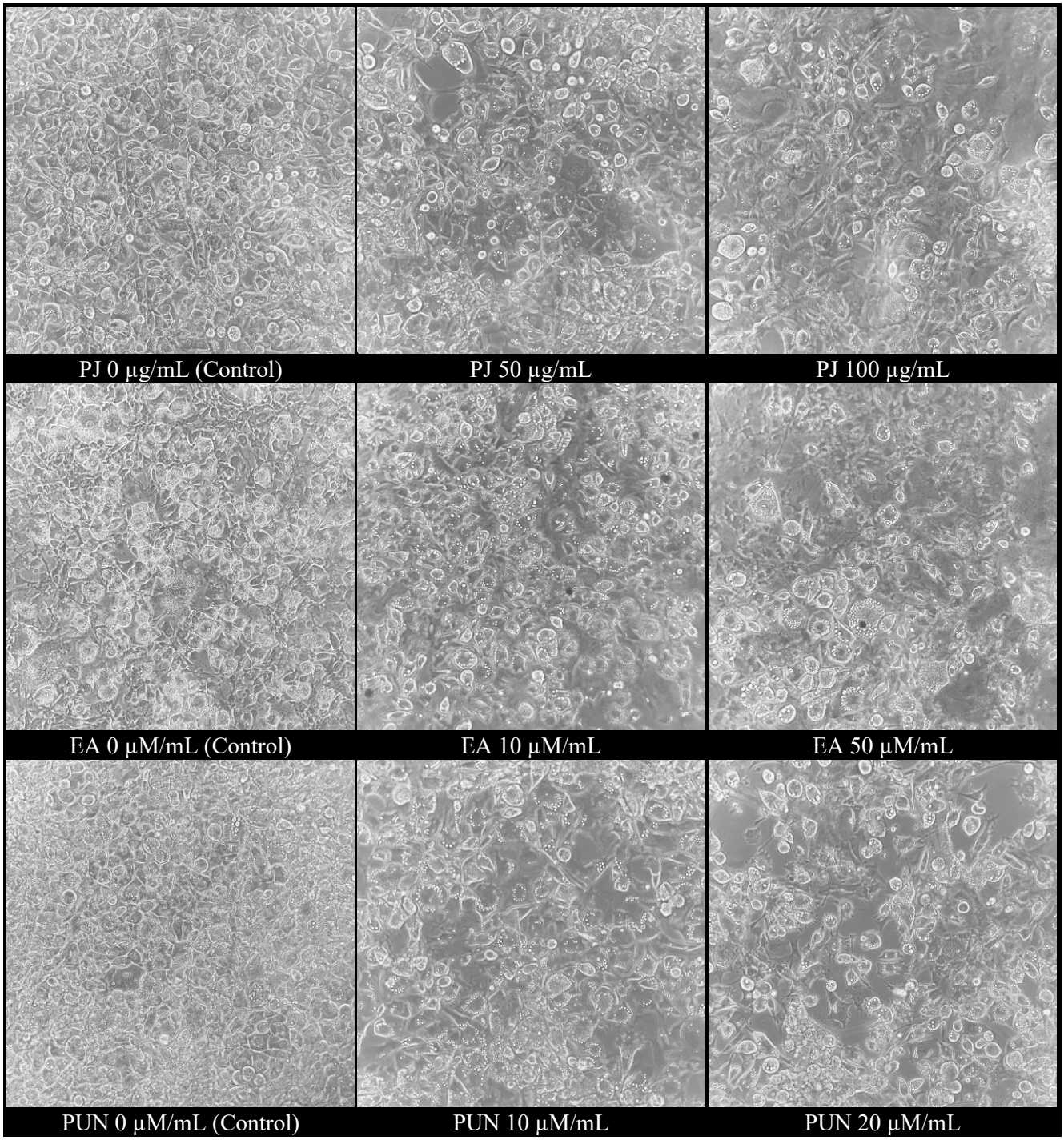


Figure 4. Dipeptidyl peptidase-4 inhibition by pomegranate polyphenols, PJ and sitagliptin

3.2. 3T3-L1 cell culture:

We next investigated the effect of PJ and its polyphenols on the differentiation of 3T3-L1 preadipocytes into adipocytes. As illustrated in the Figure 5, untreated 3T3-L1 cells showed enlarged adipocytes. However,

when the differentiation medium was supplemented with various concentrations of either PJ or the isolated compounds this supplementation produced a decrease in adipocyte differentiation in a dose-dependent fashion. In agreement with this reduced adipogenesis, a decrease of TG accumulation was observed as the concentration of the tested compounds increased in the culture medium (Figure 6). This delipidating effect was especially effective in the treatments with punicalagin and urolithin-A, both decreasing by half the TG content of the adipocytes, with IC_{50} of 0.027 and 0.002 mg/mL, respectively.



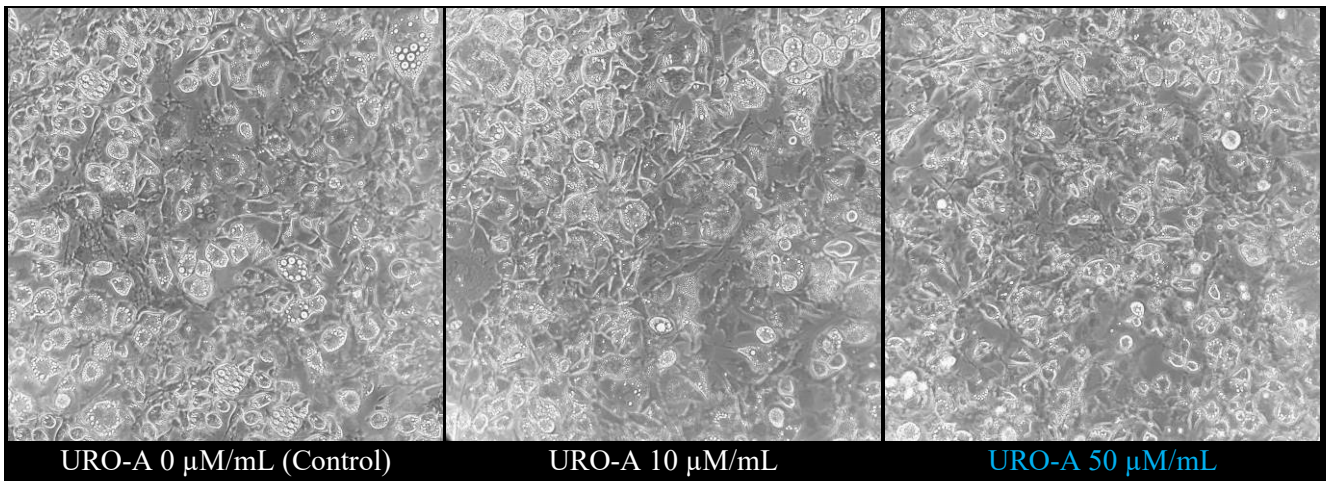


Figure 5. Microscope images (x20) of 3T3-L1 cells treated with PJ (pomegranate juice), EA (ellagic acid), PUN (punicalagin) and URO-A (urolithin A) at different doses in the last day of differentiation. Original images can be found in supplementary material.

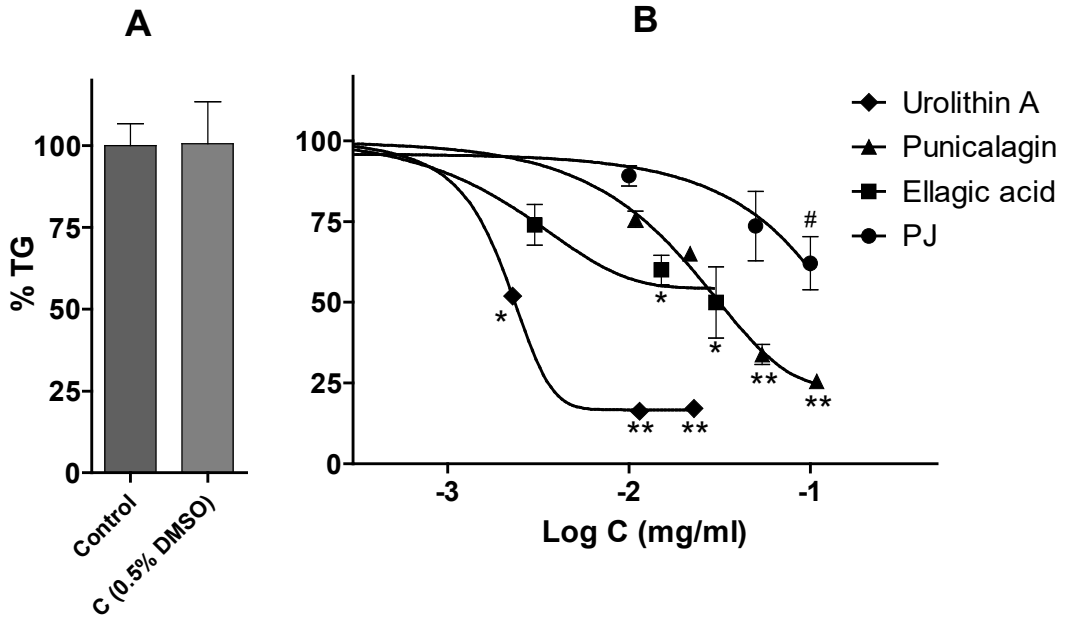


Figure 6. A. Percentage of triglycerides in 3T3-L1 cells, with and without DMSO. B: Inhibition of the formation of triglycerides in 3T3-L1 cells treated by PJ and its main compounds. The TG 100% is equivalent to 2.3 µg TG/µg protein. Significant differences were calculated by Student's *t*-test comparing control activity with PJ: # (p <0.05); and control with 0.5% DMSO with PUN, EA and URO-A: * (p <0.05) and ** (p <0.01).

3.3. Gene expression

Lastly, we measured the mRNA levels of some key adipogenic genes, markers of adipocyte differentiation. mRNA levels of adiponectin, PPARγ, GLUT4 and FABP4 were measured in 3T3-L1 derived adipocytes differentiated in the presence or absence of PJ or the isolated compounds.

When normalized by actin and expressed as percent control a clear trend was observed where all tested products decreased the gene expression in a dose-dependent manner, specially punicalagin and urolithin A (figure 7). For statistical comparisons, each sample was compared to its respective control, since the experiments were performed in different batches. It is worth mentioning that urolithin A abolished the expression of the four genes, with a significance of $p < 0.001$.

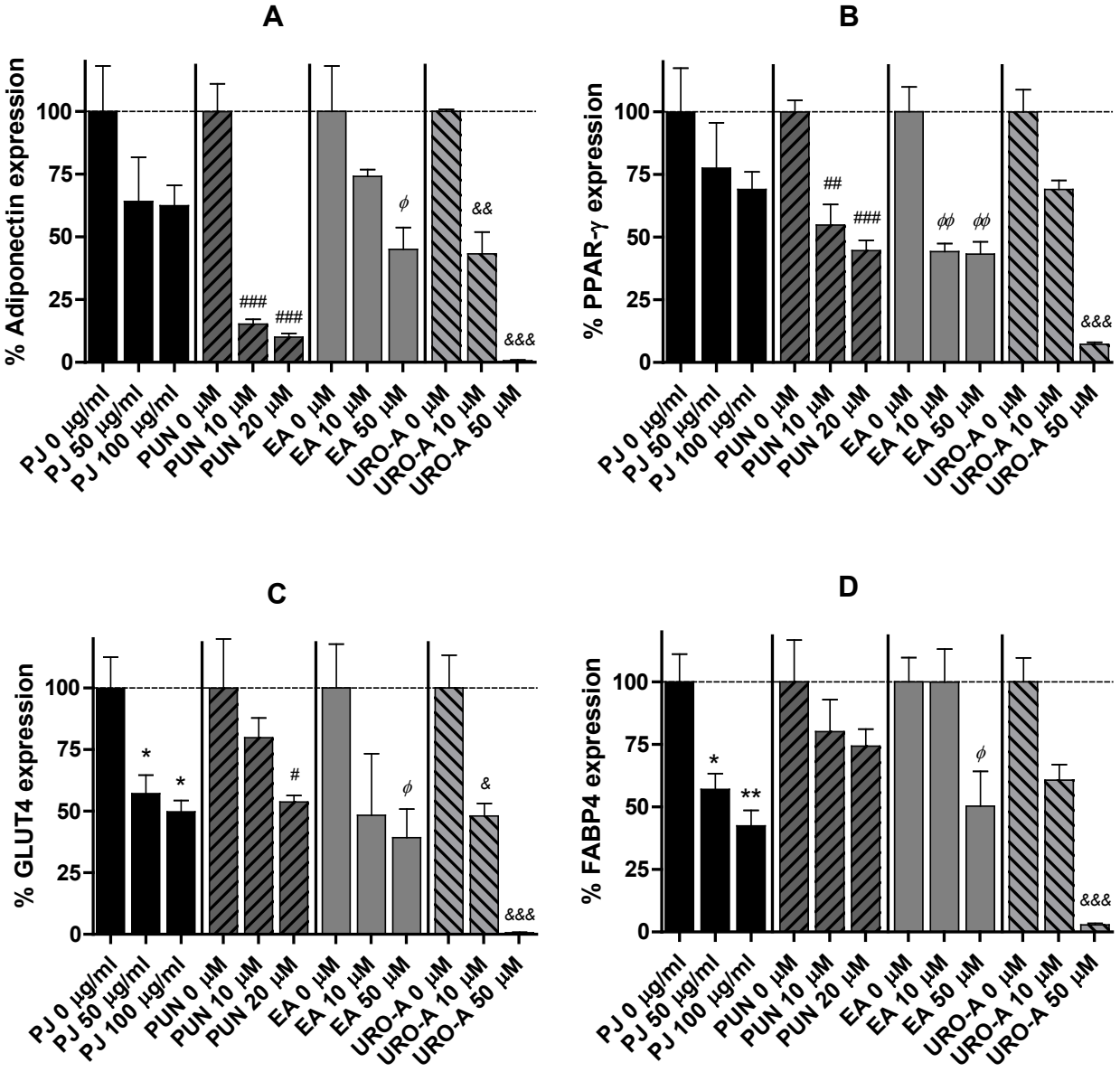


Figure 7. Gene expression normalized with actin. A: Adiponectin. B: PPARγ. C: GLUT4. D: FABP4.

Significant differences were calculated by Student's *t*-test comparing each control activity with each of its

samples. PJ * (p <0.05) and ** (p <0.01); PUN $^{\#}$ (p <0.05), $^{\#\#}$ (p <0.01) and $^{\#\#\#}$ (p <0.001); EA ^{ø} (p <0.05), ^{øø} (p <0.01) and ^{øøø} (p <0.001); URO-A $^{\&}$ (p <0.05), $^{\&\&}$ (p <0.01) and $^{\&\&\&}$ (p <0.001).

To further assess the effect of pomegranate polyphenols at the transcriptional levels, the mRNA levels of the selected genes were also normalized by FABP4. This allowed us to zero in the effects on adipocytes as, unlike actin which is ubiquitously expressed, AFABP is almost exclusively expressed in mature adipocytes (figure 8). Although some statistical significances did vary, this approach yielded essentially the same trends previously observed, highlighting the antiadipogenic effects of the studied compounds.

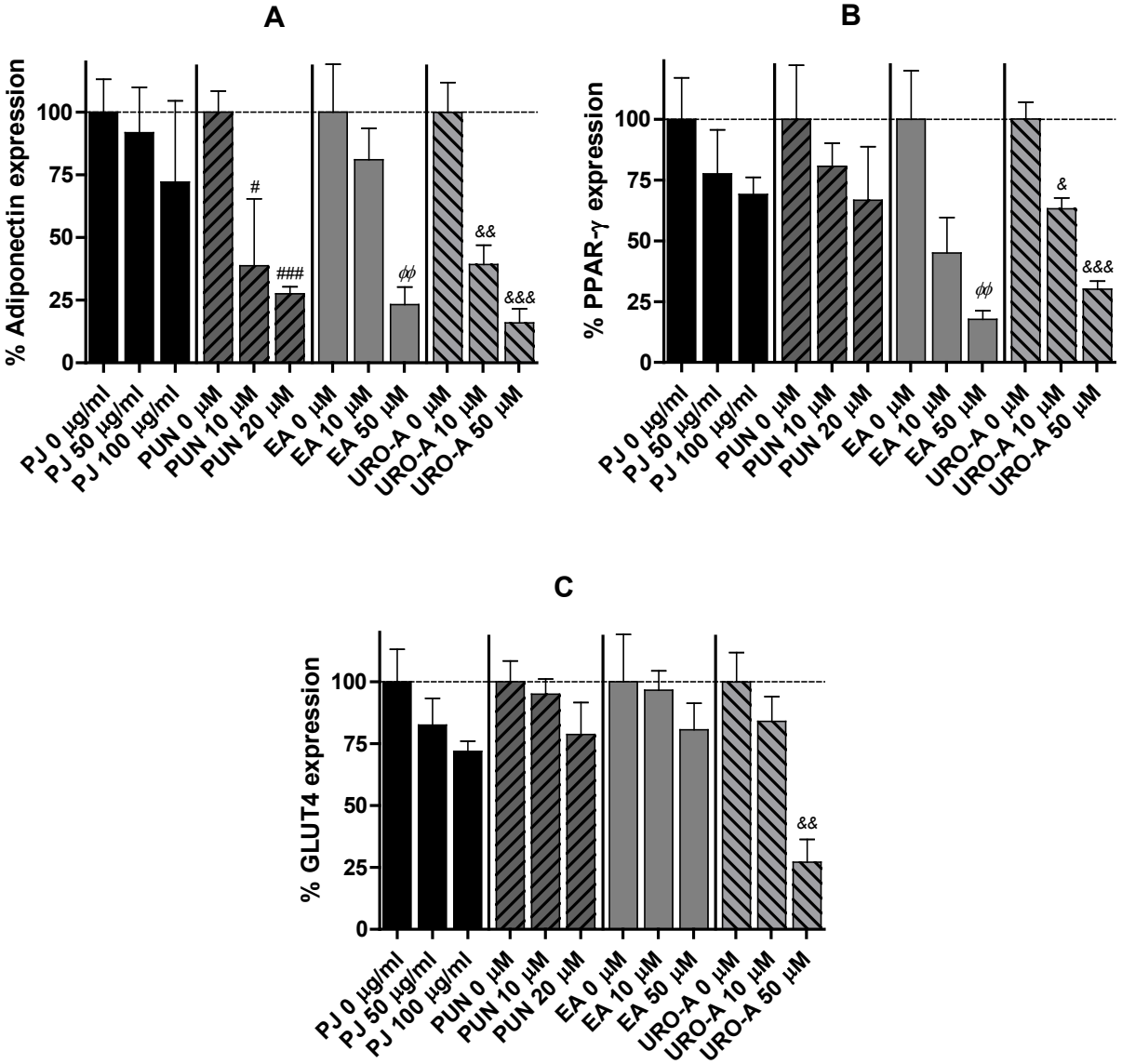


Figure 8. Gene expression normalized with FABP4. A: Adiponectin. B: PPARγ. C: GLUT4. Significant differences were calculated by Student's *t*-test comparing each control activity with each of its samples. PUN $^{\#}$ (p <0.05) and $^{\#\#\#}$ (p <0.001); EA ^{øø} (p <0.01); URO-A $^{\&}$ (p <0.05), $^{\&\&}$ (p <0.01) and $^{\&\&\&}$ (p <0.001).

4. Discussion

Several studies have shown that dietary polyphenols exert a protective role against oxidative stress and free radical damage (Del Rio et al., 2013) by their ability to eliminate or neutralize free radicals (Prior, 2003). Pomegranate is also appreciated and used in Unani medicine and Islamic countries for the treatment of metabolic disorders such as diabetes (Ahmed et al., 2013; Li et al., 2007). The most important polyphenols in pomegranate juice are punicalagin and ellagic acid. Punicalagin are exclusive to pomegranate but ellagic acid is present in other fruits as black raspberry and blackberry (Wada and Ou, 2002), cloudberry (Määttä-Riihinen et al., 2004), and red raspberry and strawberry (Mattila and Kumpulainen, 2002), and nuts as chestnut (De Vasconcelos et al., 2007) and walnut (Li et al., 2006). Moreover, it is known that pomegranate ellagitannins are metabolized in the digestive tract and transformed into urolithins by the intestinal microbiota, having a better bioavailability than the former polyphenols (González-Sarrías et al., 2015; Kang et al., 2016; Seeram et al., 2006).

This study shows that punicalagin, ellagic acid and urolithin A have the ability to inhibit enzymes in relation with the metabolism of carbohydrates and triglycerides, such as α-GLU, DPP-4 and lipase. In addition, treatments of the 3T3-L1 cell line during differentiation with these polyphenols showed the ability of these compounds to inhibit adipogenesis as well as the ability to decrease triglyceride accumulation. It was also confirmed that they have the ability to modulate the gene expression of genes that regulate the metabolism of glucose and fatty acids, such as GLUT4, FABP4, adiponectin and PPARγ genes, and commonly used as markers of adipocyte differentiation.

A therapeutic approach for the treatment of diabetes is to slow glucose uptake through the inhibition of enzymes such as α-GLU in the digestive tract. In addition, reducing the absorption of glucose may also help prevent obesity. Punicalagin, ellagic acid and urolithin A showed a very similar dose-dependent inhibition, and much greater than the reference inhibitor for α-GLU, acarbose. This α-GLU inhibition had been already reported by punicalagin (Bellesia et al., 2015) and ellagic acid (Bellesia et al., 2015; Lee et al., 2017; Mineo et al., 2015), but it is the first time that urolithin A has shown this activity.

Another enzyme present in the digestive tract is lipase. The inhibition of this enzyme by orlistat prevents the break down, and therefore, subsequent absorption of the lipids contained in the diet, being a current treatment against obesity. The polyphenols tested also inhibited this enzyme with a lower IC_{50} than orlistat,

reference inhibitor, but reaching better percentages of inhibition, 100%, against 75% of the orlistat. This dose dependent inhibitory activity of punicalagin and ellagic acid, was already proved by previous studies (C.-J. Lee et al., 2017; Mineo et al., 2015), however, it is the first time reported for urolithin A. PJ has previously showed α-GLU and lipase inhibition (Ambigaipalan et al., 2016; Colantuono et al., 2016), and other plants with content in ellagitannins has also shown activity against these enzymes, such as *Myrcia palustris* DC. (Wubshet et al., 2015), *Crataegus azarolus* (Abu-Gharbieh and Shehab, 2017) and *Adiantum capillus-veneris* L. (Kasabri et al., 2017).

Another approach for treating diabetes in recent years is the modulation of GLP-1 levels. This peptide is secreted by the enteroendocrine L cells, and stimulates insulin secretion which depends on the concentration of glucose in the blood. GLP-1 is inactivated by DPP-4, a circulating catabolic enzyme, decreasing its half-life. DPP-4 inhibitors are currently used in association with metformin for the treatment of type 2 diabetes. Punicalagin, ellagic acid and urolithin A inhibited in a dose dependent manner the DPP-4 enzyme; however, the IC_{50} values for these polyphenols were higher than the antidiabetic drug sitagliptin. To the best of our knowledge, it is the first time that this enzyme is reported to be inhibited by these polyphenols and the metabolite urolithin A. Nonetheless, some studies have already shown that non-nutritional dietary factors such as polyphenols may affect GLP-1 levels (Tsuda, 2015).

The ability of PJ and its two major polyphenols to inhibit the adipogenesis has already been demonstrated in previous studies (Les et al., 2017). This fact agrees with the decrease in the number of adipocytes observed in the treatment of the 3T3-L1 cell line with the different polyphenols. In addition, it has also recently been reported that urolithin A suppresses adipogenesis and decrease the triglycerides accumulation in human adipogenic stem cells (Kang et al., 2016). This activity has been shown in our study treating 3T3-L1 cells with PJ polyphenols, being able to reduce triglycerides accumulation compared to untreated cells, highlighting the greater activity of urolithin A compared to punicalagin, ellagic acid and PJ.

After the treatment of 3T3-L1-derived adipocytes, we demonstrated a dose-dependent inhibition of adipogenesis. The inhibitory role of PJ and its metabolites was reflected by the reduced mRNA levels of the key adipogenic genes PPARγ and FABP4. Interestingly, both actin and FABP4 normalization yielded similar results, lending a strong support to the antiadipogenic effects of pomegranate polyphenols. It is worth noting that urolithin A was the compound with the highest inhibitory effects, which lead us to hypothesize that this metabolite bears most of the antiadipogenic ability of PJ. Urolithin A has already shown inhibitory activity

reducing protein expression of FABP4 and PPARγ (Kang et al., 2016), but is the first time where it is reported the ability to inhibit gene expression of adiponectin and GLUT4 proteins. Ellagic acid has shown the capacity to modulate the PKC-R/ERK/PPARγ/NF-κB pathway (Kuo et al., 2011) and reduced fat accumulation by down-regulating adipogenic markers such as PPARγ (Woo et al., 2015). However, other studies have shown punicic acid, a major bioactive fatty acid found in pomegranate seed, increased adiponectin secretion and upregulated GLUT4 expression and translocation in adipocytes (Anusree et al., 2014). Pomegranate phytochemicals could have interest in diabetes due to the fact punicic acid is involved in the upregulation gene signalling of adiponectin, GLUT4 and PPAR (Irudayaraj et al., 2016) and polyphenols may act as α-GLU, DPP-4 inhibitors. All these compounds might act synergistically when pomegranate is consumed. Moreover, the downregulation of the adiponectin, PPARγ, GLUT4 and FABP4 genes, the reduction of triglycerides accumulation in adipocyte-like cells and the inhibition of lipase by PJ and these polyphenols, and mainly by urolithin A, could be interesting in obesity prevention.

According to the manuscript, all the effects observed for PJ, and particularly urolithin A, support scientific evidence at a molecular level for preventing metabolic diseases but further research is required for well-established clinical benefits.

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Author contribution

Francisco Les carried out the experiments and wrote the first draft of the manuscript. Jose Miguel Arbonés-Mainar designed the experiments with the 3T3-L1 cells and interpreted the results on gene expression. Marta Sofía Valero designed and supervised the work. Víctor López designed the study, corrected the paper and set up the in vitro enzymatic experiments.

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