Pomegranate ellagitannins inhibit α-glucosidase activity *in vitro* and reduce starch digestibility under simulated gastro-intestinal conditions

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Abstract

Pomegranate extract was tested for its ability to inhibit α-amylase and α-glucosidase activity. Pomegranate extract strongly inhibited rat intestinal α-glucosidase *in vitro* whereas it was a weak inhibitor of porcine α-amylase. The inhibitory activity was recovered in an ellagitannins-enriched fraction and punicalagin, punicalin and ellagic acid were identified as α-glucosidase inhibitors (IC_{50} of 140.2, 191.4 and 380.9 μmol/L, respectively). Kinetic analysis suggested that the pomegranate extract and ellagitannins inhibited α-glucosidase activity in a mixed mode. The inhibitory activity was demonstrated using an *in vitro* digestion system, mimicking the physiological gastro-intestinal condition, and potatoes as food rich in starch. Pre-incubation between ellagitannins and α-glucosidase increased the inhibitory activity, suggesting that they acted by binding to α-glucosidase. During digestion punicalin and punicalagin concentration decreased. Despite this loss, the pomegranate extract retained high inhibitory activity. This study suggests that pomegranate ellagitannins may inhibit α-glucosidase activity *in vitro* possibly affecting *in vivo* starch digestion.

Keywords: pomegranate, ellagitannins, starch digestion, diabetes, mass spectrometry

Introduction

High intakes of fruit and vegetables have been associated with a lower incidence of chronic diseases including diabetes, cardiovascular diseases and cancer (Boeing et al. 2012). It is now widely accepted that the protection supplied by fruit and vegetables against diseases is due to the presence of various bioactive compounds. Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites found in plants. Various *in vitro* and *in vivo* evidence show that several (poly)phenol-rich foods are protective against chronic diseases, including cardiovascular disease, neurodegeneration, and cancer (Del Rio et al. 2013).

One of the principal topics concerning the beneficial effects of (poly)phenols is their bio-availability and metabolic fate. Most of dietary phenolic compounds are subjected to extensive metabolism prior and after the absorption such that, with very few exceptions, only metabolites of the parent compounds enter the circulatory system (Del Rio et al. 2013). As a result, the gastrointestinal tract could be the location for the health benefits derived from a diet rich in (poly)phenols. Phenolic compounds might exert direct protective effects in the gastrointestinal tract, by scavenging reactive oxygen species (Halliwell et al. 2000). The inhibition of intestinal carcinogenesis by red wine (poly)phenols, grape seed extract and berries has been demonstrated in cell lines, animal model systems and humans (Dolara et al. 2005; Kaur et al. 2006; Adhami et al. 2009). In addition, (poly)phenols are able to inhibit some intestinal digestive enzymes such as lipase and glucosidases, modulating nutrients bioavailability and resulting in a beneficial effect on obesity and blood glucose control (McDougall and Stewart 2005).

The prevalence of type II diabetes is rising exponentially and particular non-insulin-dependent diabetes mellitus is intimately associated to cardiovascular complications as a consequence of post-prandial hyperglycemic condition (Nathanson and Nyström 2009). Inhibitors of intestinal α-glucosidase enzymes retard the rate of carbohydrate digestion, contributing to reduce post-prandial hyperglycemia (Krentz and Bailey 2005). The use of commercial α-glucosidase inhibitors (acarbose, miglitol and voglibose) is limited by their gastro-intestinal intolerability and high cost. One intriguing approach to control hyperglycemia could be its prevention by phytochemicals present in the diet. Several reports have been published in recent years showing that berry, red wine and green tea (poly)phenols are able to inhibit *in vitro* intestinal glucosidases, potentially suggesting their efficacy in an effective management of diabetes mellitus (Boath et al. 2012a; Kwon et al. 2008). Pomegranate (*Punica granatum* L.) is a rich source of phytochemicals, mainly anthocyanins, ellagitannins (punicalin, punicalagin, pedunculagin) and ellagic acid with antioxidant, anti-cancer and cardiovascular protective activities (Medjakovic and Jungbauer 2013; Usta et al. 2013). Pomegranate has also been studied for its anti-diabetic properties. Pomegranate juice supplementation significantly reduced post-prandial blood glucose but not triacylglycerols and cholesterol levels in streptozotocin-induced diabetic mice fed with a high-fat diet (Betanzos Cabrera et al. 2011). Indeed, *Punica granatum* flower extract was able to reduce post-prandial hyperglycemia in Zucker diabetic fatty rats (Li et al. 2005). A possible explanation for these observations is that pomegranate juice possesses α-glucosidase or α-amylase inhibitors able to attenuate the post-prandial increase in glycemia. A reduction of α-glucosidase activity was observed in the saliva of healthy humans after the consumption of pomegranate extract during an intervention study (Di Silvestro et al. 2009). The present study tested a pomegranate (poly)phenol-rich extract for α-glucosidase and α-amylase inhibitory activity to determine its potential mechanism of action as hypoglycemic agent. Pomegranate extract was subsequently fractionated with the aim to identify compounds that may influence the enzymatic activities. Finally, the inhibitory effect on carbohydrate hydrolysis of pomegranate extract was tested against a real food system (potatoes) using an *in vitro* digestion model.

Methods

Materials

α-Glucosidase (EC 3.2.1.20) from rat intestinal acetone powder, porcine pancreatic α-amylase (EC 3.2. 1.1), bile salts (mixture of sodium cholate and sodium deoxycholate), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, potato starch, *p*-nitrophenyl α-D-glucoside (PNP-gluc), acarbose and Sephadex LH-20 were purchased from Sigma Chemical Co. (Milan, Italy). All the other chemicals for enzymatic reactions and digestion procedure were obtained from Sigma Chemical Co. (Milan, Italy). Formic acid, acetonitrile, ethanol and methanol for column chromatography, HPLC and LC-MS analysis were from Carlo Erba (Milan, Italy). Standard compounds for HPLC analysis were also supplied by Sigma Chemical Co. (Milan, Italy) except punicalin that was purified from pomegranate extract. Sephadex C-18 columns (quantity of sorbent 10000 mg) were supplied by Alltech (Deerfield, IL). Pomegranate juice (Azienda Montana Achillea; Paesana, Cn, Italy) was purchased from a local supermarket (Reggio Emilia, Italy) and was 100% pure pomegranate juice.

Sample preparation and total (poly)phenol determination

Pomegranate juice (poly)phenol-rich extract was obtained using C18 solid-phase extraction (Verzelloni et al. 2007). Columns were pre-conditioned with 60 mL of methanol and subsequently with 40 mL of 0.1% formic acid in water. The pomegranate juice was loaded (20 mL) and 60 mL of 0.1% formic acid was used to elute unbound materials (free sugars, organic acids and vitamin C). The bound materials, containing pomegranate (poly)phenols, were eluted with 60 ml of methanol. The solvent was removed by a rotary evaporator to near dryness and then freeze-dried. The pomegranate extract was tested for its ability to inhibit the activity of α-glucosidase and α-amylase.

Total (poly)phenols in pomegranate juice and extract were determined using the Folin-Ciocalteau method (Singleton et al. 1999). The total phenolic content was expressed in mmol/L of ellagic acid equivalents, using ellagic acid as standard at concentrations ranging between 20 and 1000 mmols/L. The choice of the standard was carried out considering that ellagitannins (which are built up with ellagic acid units) are the most predominant phenolic components present in pomegranate juice (Fischer et al. 2011).

Amylase assay

Amylase assay was carried out as reported by McDougall et al. (2005) using porcine pancreatic α-amylase and soluble potato starch as a substrate. The reaction was performed in 20 mmol/L sodium phosphate buffer pH 6.9 containing 6.7 mmol/L NaCl. For the reaction, 0.1 mL of 2 U/mL amylase solution (one unit of amylase is defined as the quantity of enzyme that releases 1.0 mg of maltose from starch in 3 minutes at pH 6.9 at 20°C) was mixed with 0.9 mL of sodium posphate buffer or different concentrations of pomegranate extract dissolved in the sodium posphate buffer. After 10 min at 37°C, 1 mL of 1% starch solution (dissolved in the sodium posphate buffer) was added and the reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 1 mL of dinitrosalicylic acid solution and boiling for 15 min in a water bath. Enzyme activity was quantified by measuring the mg of maltose released from starch by reading at 540 nm. To calculate the IC_{50} value, the enzyme activity was determined in the presence of pomegranate extract with phenols concentrations ranging from 150 to 3000 µmol/L. The IC_{50} is defined as the concentration of phenolics required to inhibit 50% of the enzymatic activity.

α-Glucosidase assay

The enzyme α-glucosidase was extracted from rat intestinal acetone powder and assayed according to Oki et al. (1999). The rate of release of *p*-nitrophenol (PNP) from PNP-gluc was measured at 37 °C after incubation for 20 min in presence of 0.01 U/mL of rat intestinal α-glucosidase (one unit of α-glucosidase is defined as the quantity of enzyme that releases 1.0 μmol of PNP from PNP-gluc per minute at pH 6.8 at 37°C). For the reaction, 0.1 mL of 0.2 U/mL of rat intestinal α-glucosidase was pre-incubated with 0.9 mL of buffer (potassium phosphate buffer 67 mmol/L, pH 6.8) or different concentration of pomegranate extract dissolved in buffer. After 10 min at 37°C, 1 mL of substrate solution (containing 1 mmol/L PNP-gluc and 0.2 mmol/L of glutathione dissolved in potassium phosphate buffer) was added and the reaction mixture was incubated at 37°C for 20 min. The reaction was terminated by adding 4 mL of 100 mmol/L sodium carbonate solution. Enzyme activity was quantified by measuring the μmol of PNP released from PNP-gluc by reading at 400 nm. To determine the IC_{50} value, the enzyme activity was determined in the presence of pomegranate extract with phenols concentrations ranging from 150 to 3000 μmol/L. The IC_{50} is defined as the concentration of phenolics required to inhibit 50% of the enzymatic activity.

Pomegranate juice (poly)phenol-rich extract fractionation

To identify compounds responsible for the inhibitory activity, pomegranate extract was fractionated using Sephadex LH-20 with the method adapted from the *Tannin Handbook* (available at www.users.muohio.edu/hagermae/tannin.pdf). Sorption to Sephadex LH-20 in aqueous ethanol and selective de-binding with aqueous acetone is an established method for separating tannins from non-tannin phenolics). Briefly, after column preconditioning with 80% ethanol, the pomegranate extract in 80% ethanol was applied to the column. The unbound material (anthocyanins and other monomeric phenolic compounds) was collected after washing with three volumes of 80% ethanol. The bound fraction (ellagitannins) was eluted with three volumes of 50% acetone. Both the fractions were evaporated by a rotary evaporator to near dryness and and then freeze-dried. All the fractions were subjected to LC-ESI-MS/MS analysis and tested for their ability to inhibit the hydrolytic enzymes.

LC-ESI-MS/MS analysis

LC–MS/MS analysis were carried out according to Fischer et al. (2011) using an Agilent system 6310A Ion Trap LC-MS^n (Agilent, Waldbronn, Germany) equipped with degasser, binary gradient pump, thermo-autosampler and column oven. The MS/MS system was ion trap mass spectrometer fitted with an ESI source. Data acquisition and processing were performed using DataAnalysis software. Negative ion (ellagitannins) mass spectra of the column eluate were recorded in the range of m/z 50–1300 at a scan speed of 13,000 m/z/s. The mobile phase, solvent A (1% formic acid) and solvent B (acetonitrile), was used under binary linear gradient conditions as follows: 5-15% B (10 min), 15-25% B (20 min), 25–50% B (3 min), 50% B isocratic (4 min); with a flow rate of 1 mL/min.

For anthocyanins identification, positive ion mass spectra of the column eluate were recorded in the range of m/z 50–1300 at a scan speed of 13,000 m/z/s. The mobile phase consists of (A) formic acid 2% in HPLC water and (B) formic acid 2% in methanol HPLC grade. The following gradient was applied: 10–14% B (5 min), 14–23% B (11 min), 23–35% B (5 min), 35–40% B (14 min), 40–100% B (3 min), 100% B isocratic (3 min), 100–10% B (3 min), 10% B isocratic (4 min). The flow rate was 1 mL/min.

The nebuliser gas temperature was set at 400° C. Helium was used as collision gas at a pressure of $4x10^{-6}$ mbar.

HPLC-DAD analysis

Individual phenolic compounds were quantified using an HPLC system consisted of a Jasco HPLC system (Orlando FL, U.S.A.) equipped with a diode array detector, a reversed phase column Hamilton HxSil C18 (Hamilton, Reno, Nevada; 250mm x 4.6mm), a volumetric injector Rheodyne (Cotati, CA), and a temperature-controlled oven.
For ellagitannins quantification, the monitored wavelength was 360 nm. Identification and quantification of punicalagins A and B, ellagic acid and punicalin in samples were performed using calibration curves of the respective standards compounds. For this reason, a stock solutions of standard compounds were diluted at different concentrations and the solutions were analysed.
Anthocyanins were quantified at a wavelength of 520 nm as cyanidin-3-glucoside equivalents. The HPLC parameters were the same as reported in the previous section.

Identification of α-glucosidase inhibitors

The ellagitannins recognized by LC-MS/MS were tested for their α-glucosidase inhibitory activity. Ellagic acid and punicalagin (a mixture of A and B isomers) were obtained from Sigma Chemical Co. (Milan, Italy) as pure compounds (95% of purity degree). Punicalin was purified from pomegranate juice following the procedure reported in Aviram et al. (2008). Purified compound was evaporated by a rotary evaporator to near dryness and then freeze-dried. The purified compound was characterized by LC-ESI-MS/MS and the purity assayed with HPLC-DAD (95% of purity degree as deduced from the ratio of the peak area of the isolated compounds and total peak area at 280 nm; see supplementary figure).

For the calculation of IC_{50} values, α-glucosidase assay was carried out in the presence of variable amounts (from 10 to 500 μmol/L) of punicalin, punicalagin or ellagic acid.

In vitro gastro-intestinal digestion

The gastro-intestinal system was adapted from Tagliazucchi et al. (2012) with some modifications. Potatoes, selected as real starch-rich food, were weighed, peeled, and cooked whole in boiling water for 30 min. They were removed and cooled at ambient temperature (21°C) to be handled. Ten grams of cooked potatoes (corresponding to 1.71 g of starch) were homogenized in a laboratory blender for 1 min to simulate mastication in presence of 5 mL of simulated salivary fluid and 20 mL of different concentrations of pomegranate extract dissolved in a 0.1 M phosphate-buffer (pH 6.9). The artificial saliva consisted of a 0.1 M phosphate-buffer (pH 6.9) containing 1.336 mmol/L $CaCl_2$, 0.174 mmol/L $MgSO_4$, 12.8 mmol/L KH_2PO_4, and 23.8 mmol/L $NaHCO_3$, 2 g/L of food casein (known to be a proline-rich protein), and 150 units/L α-amylase.

In the control digestion, the pomegranate extract was omitted and the cooked potatoes (10g) were homogenized in presence of 5 mL of simulated salivary fluid and 20 mL of the 0.1 M phosphate-buffer (pH 6.9).

After 10 minutes of incubation at 37°C in a shaking bath, the pH was adjusted to 2.5 (to simulate gastric pH) with concentrated HCl and after 2 g/L of NaCl and 315 U/mL of pepsin were added. The solution was incubated at 37°C in a shaking bath at 100 rpm for 2 h. At the end of the gastric digestion, the pH was brought to 7.5 with $NaHCO_3$ (to simulate hepato-pancreatic pH) before adding 0.8 g/L of pancreatin, 5 mg/mL of bile salts and 2 mL of rat intestinal solution containing 10 U of α-glucosidase. On the basis of the added pancreatin, the amount of digestive enzymes in the intestinal fluid was 80 U/mL of α-amylase, 240 U/mL of proteases and 384 U/mL of lipase. The solution was then incubated at 37°C in a shaking bath at 100 rpm for a further 2 h.

The amount of glucose released at the end of the digestion was quantified using a hexokinase, glucose-6-phosphate dehydrogenase, phospho-glucose isomerase method (Kunst et al. 1984).

The ellagitannins were quantified by HPLC-DAD as reported in the previous section.

Statistical analysis

All data are presented as mean ± SD for three replicates for each prepared sample. The Student's t-test and ANOVA with Tukey post-hoc test was performed using Graph Pad Prism (GraphPad Software, San Diego, CA). The differences were considered significant with $P < 0.05$. The IC_{50} values were determined using nonlinear regression analysis and fitting the data with the log(inhibitor) vs. response model (Graph Pad Prism).

Results

The pomegranate juice contained 6.82 ± 0.75 mmol of ellagic acid equivalent (EAE)/L of phenolic compounds. The percentage of the recovery in the C18 bound fraction, corresponding to the pomegranate extract, was 86% of total (poly)phenols (5.87 ± 0.26 mmol of ellagic acid equivalent (EAE)/L).

Pomegranate extract was an effective inhibitor of rat intestinal α-glucosidase with an IC_{50} value of 922.8 ± 1.2 μmol of EAE equivalent/L (**Figure 1**). Acarbose inhibited α-glucosidase in a dose-dependent manner giving an IC_{50} value of 69.7 μmol/L.

On the contrary, pomegranate extract was a weak inhibitor of α-amylase. At the highest tested concentration, corresponding to a final concentration of pomegranate (poly)phenols in the assay of 3000 μmol of EAE equivalent/L, the α-amylase activity was inhibited by 42%. These results showed that pomegranate extract contained potent inhibitors of rat intestinal α-glucosidase.

The pomegranate extract was fractionated in two different fractions with Sephadex LH-20. The phenolic compounds in the two Sephadex LH-20 fractions were characterised by LC-ESI-MS/MS analysis and the individual compounds quantified by HPLC-DAD analysis. The LH-20 unbound material was pink and contained mainly anthocyanins (**Figure 2**) as delphinidin 3,5-diglucoside (19.6 ± 0.4 μmol/L), cyanidin 3,5-diglucoside (57.5 ± 1.2 μmol/L), pelargonidin 3,5-diglucoside (11.6 ± 0.2 μmol/L), delphinidin 3-glucoside (11.1 ± 0.2 μmol/L) and cyanidin 3-glucoside (12.8 ± 0.4 μmol/L), low levels of ellagitannins and unidentified flavonols. Instead the LH-20 bound material was brown and contained the majority of ellagitannins (**Figure 3**).

Enzymatic analysis showed that only the LH-20 bound fraction caused inhibition of α-glucosidase, whereas the LH-20 unbound fraction did not show any inhibitory activity, even at the highest tested concentrations. It is interesting to note that also the majority of the α-amylase inhibitory activity was recovered in the LH-20 bound fraction, with only a marginal activity found in the LH-20 unbound material.

The retention times, concentration (µmol/L) and mass spectral characteristics of the ellagitannins are specified in **Table 1**.

Punicalin is the major ellagitannins (peak 6; **figure 3A**) found in the pomegranate extract; this compound present an [M-H]- ion at *m/z* 781 and fragments at *m/z* 601 and 602 for the loss of gallagic acid moiety.

Punicalagin showed an [M-H]- ion at *m/z* 1083 but it can be also detected as doubly charged ion species at *m/z* 541. The fragment at *m/z* 601 in MS/MS experiment showed the loss of a gallagic acid moiety and a fragment with *m/z* 781 was observed equivalent to the $[M-H]^-$ ion of punicalin. The presence of the two isomers A and B (peaks 7 and 8; **figure 3A**) was confirmed by the different retention times of the commercial standard isomers.

The compound eluting at 15.0 min exhibited an $[M-H]^-$ ion at *m/z* 783. The loss of water moiety and ellagic acid (*m/z* 301) in MS/MS experiment produced fragments at *m/z* 765 and *m/z* 481, respectively. Based on this fragmentation pathway and a previous study (Okuda et al. 1983) this compound was identified as bis-HHDP-hexoside (pedunculagin A; peak 9; **figure 3A**).

The compound present in the peak 10 (**figure 3A**) eluted at 18.4 min and exhibited an $[M-H]^-$ ion at *m/z* 951. In MS/MS experiment produced fragments at *m/z* 933 and 934. Furthermore, fragments at *m/z* 915 were obtained from the loss of water moiety from principal fragment (m/z 933) and the ion at *m/z* 897 by dehydration. This compound was tentatively identified as granatin B based on the fragmentation pattern reported in previous study (Fischer et al. 2001).

The compound which eluted at 19.7 min with fragment at *m/z* 463 was identified as ellagic acid-hexoside (peak 11; **figure 3A**). This compound produced fragments at *m/z* 300, 301, 302 in MS/MS experiment, typical m/z fragments of ellagic acid. Ellagic acid-hexoside has previously reported in pomegranate juice and arils (Fischer et al. 2001).

The last identified compound was ellagic acid (peak 12; **figure 3A**). The aglycone moiety (*m/z* 301) produced characteristic fragments at *m/z* 229, 201 and 185 in MS/MS experiment. Ellagitannins and ellagic acid were therefore identified as the α-glucosidase inhibitors present in the pomegranate extract. The IC_{50} values of the individual ellagitannins, revealed that punicalagin was the most effective inhibitor of α-glucosidase (IC_{50} of 140.2 ± 1.1 μmol/L) followed by punicalin and ellagic acid (IC_{50} of 191.4 ± 1.3 μmol/L and 380.9 ± 3.5 μmol/L, respectively).

To gain more information about the role of each identified ellagitannins in the α-glucosidase activity inhibition, their contribution ratio was calculated by dividing the power of inhibitory activity of each identified compound (calculated by dividing the amount of each single compound in the extract in μmol/L by its IC_{50} value in μmol/L) with that of the pomegranate extract (calculated by dividing the total (poly)phenolic content of the extract in μmol/L by its IC_{50} value in μmol/L) (Toshima et al., 2010). The obtained value was than multiplied by 100 to estimate the contribution ratio as %. For example, the contribution ratio of punicalagin was calculated as follows: (232.2/140.2)*100/(5870/922.8) = 26%. The same calculation for punicalin and ellagic acid provides contribution ratio values of 54 and 3%, respectively. The data reported clearly indicated that the α-glucosidase inhibitory activity of pomegranate extract was due to punicalin and punicalagin with a minor contribution of ellagic acid.

Kinetic analysis and mechanism of inhibition

In the original assay, the pomegranate extract was mixed with α-glucosidase and buffer, pre-incubated for 10 min and the reaction started by the addition of the substrate. If the order of addition of components was changed and the reaction started by the addition of the enzyme rather than the substrate, then the pomegranate extract was less effective (**Figure 4A**). The same effect was observed when different concentrations of ellagic acid were pre-incubated for 0, 5, 10, 30 or 60 min with α-glucosidase (**Figure 4B**). This results suggested that pomegranate ellagitannins interacted directly with the α-glucosidase.

The ellagitannin punicalagin as well as the pomegranate extract were selected as test inhibitors for the kinetic analysis. All the tested samples reduced the V_{max} and increased K_M of α-glucosidase (**Table 2**). These results suggested a mixed-type inhibition with respect to substrate concentration.

Effect of pomegranate extract on potato starch hydrolysis during in vitro gastro-intestinal digestion

The ability of the pomegranate extract to inhibit starch hydrolysis was assessed using a real food during simulated gastro-intestinal conditions. Cooked potatoes was firstly subjected to mastication, in presence of simulated salivary fluid which contained 150 units/L of α-amylase. After 10 minutes, the bolus was subjected to consecutive gastric (2 h) and intestinal (2 h) digestion, in presence of 80 units/mL of α-amylase and 370 units/L of α-glucosidase.

At the end of the gastro-intestinal digestion, in absence of the pomegranate extract, the amount of released glucose was 199.5 ± 2.12 mg/g of potato starch. The addition of the pomegranate extract in the digestive system produced a decrease in the amount of released glucose at the end of the gastro-intestinal digestion of 18 and 44% when the digestion was carried out with 2.35 or 4.7 mmol/L of total (poly)phenols, respectively. Control experiments carried out without enzymes showed that there was no hydrolysis of potato starch.

The behaviour of the ellagitannins during simulated gastro-intestinal digestion of potatoes was followed with HPLC-DAD. The results are detailed in **Table 3**. The concentration of the ellagitannins punicalin and punicalagin decreased by 22.6 and 30.9% after mastication and by 36.8 and 61.6% after pancreatic digestion, respectively. The amount of ellagic acid increases to 142.8 and 234.2% after mastication and pancreatic digestion, respectively.

Discussion

This is the first report showing that pomegranate juice (poly)phenolic extract is a potent inhibitor of *in vitro* carbohydrate digestion. Pomegranate extract strongly inhibited the rat intestinal α-glucosidase activity *in vitro*.

The ability of the pomegranate (poly)phenolic-rich extract to inhibit the starch hydrolysis was also demonstrated using an *in vitro* digestion system, mimicking the physiological gastro-intestinal condition, and potatoes as food rich in starch.

A variety of food (poly)phenolic extracts have been shown to inhibit α-amylase and α-glucosidase activities *in vitro*. Rat intestinal α-glucosidase inhibitory activity of pomegranate extract (IC_{50} value of 278 μg/mL) is lower than that of acarbose (IC_{50} of 45 μg/mL), anthocyanins-rich berry extracts (such as blueberry, blackcurrant, rowanberry, and strawberry; IC_{50} values from 18 to 42 μg/mL), and black tea (IC_{50} of 64 μg/mL) (McDougall et al., 2005, Koh et al. 2010). However, the *in vitro* inhibitory activity of pomegranate extract was similar to that of green tea (IC_{50} value of 297 μg/mL) which has been found to be effective in reducing postprandial blood glucose level *in vivo* (Tang et al. 2013).

The inhibitory activity against both the enzymes was assigned to ellagitannins, especially punicalin and punicalagin. The comparison of the IC_{50} values against rat intestinal α-glucosidase of punicalagin and punicalin (140.2 and 191.4 μmol/L, respectively) with that of other (poly)phenols revealed that these compounds are effective as theaflavin digallate (IC_{50} of 165 μmol/L, Koh et al. 2010) and diacilated anthocyanins (IC_{50} of 200 μmol/L, Matsui et al. 2002). Pomegranate ellagitannins are more effective than green tea catechins (Koh et al. 2010), and flavonols (Tadera et al. 2006). Pomegranate ellagitannins are less effective than acarbose (IC_{50} of 69.7 μmol/L).

Punicalagin, despite its lower IC_{50} value against rat intestinal α-glucosidase, was not the most important contributor to the inhibitory activity (26% of contribution). In contrast, punicalin was estimated to be the main contributor to pomegranate extract α-glucosidase inhibition (54% of contribution) owing to its higher content in the extract. The total contribution ratio of all identified ellagitannins in this study was 83%, suggesting that some unidentified compounds with α-glucosidase inhibitory activity can be present in the pomegranate extract or that synergic effects should be considered.

Kinetic analysis suggested that pomegranate extract, and ellagitannins inhibited α-glucosidase activity in a mixed mode. The pre-incubation and the order of addition experiments indicate that ellagitannins influence α-glucosidase activity via their ability to bind proteins (Wang et al. 2013). The non-specific binding of ellagitannins with α-glucosidase may alter the structure of the enzyme by reducing the velocity of the catalysis and the accessibility to the active site of the substrate.

Most of the studies previously published on the inhibitory activity of (poly)phenols or (poly)phenols-rich extract against α-amylase and α-glucosidase were carried out using enzymatic assay that did not represent the physiological conditions of the gastro-intestinal tract. One of the most important criticisms in employing the enzymatic assay is the use of starch solution or synthetic substrate solution instead of real food. The importance of utilizing real food lies in the presence of additional molecules (such as proteins, lipids and fibers), other than starch, that may impede the effect of (poly)phenols on the enzymes. An additional criticism is related to the fact that phenolic compounds are somewhat unstable under real or simulated gastro-intestinal conditions. For example it has been shown that anthocyanins are degraded in the pancreatic media (Liu et al. 2014) whereas ellagitannins may undergo partial breakdown in the gastro-intestinal tract (Larrosa et al. 2010). To overcome this point, we tested the ability of pomegranate (poly)phenols to inhibit the carbohydrate hydrolysis during simulated digestion of potatoes. Results show that the pomegranate extract is able to inhibit in a concentration dependent manner potato starch digestion under *in vitro* gastro-intestinal conditions. Despite all the limitations of the model system (static model, glucosidase not bound to the enterocyte membrane), our results allow us to infer that pomegranate (poly)phenols may be effective inhibitors of starch digestion also *in vivo* by inhibiting the activity of α-glucosidase. Our results show that a portion of pomegranate juice (200 ml) is able to inhibit the starch hydrolysis by about 50% during the digestion of a portion (100 g) of potatoes. As already observed, ellagitannins are not stable under gastro-intestinal condition (Larrosa et al. 2010). We found a decrease in the concentration of the ellagitannins punicalin and punicalagin by 22.6 and 30.9% after mastication. These decreases may be due to the irreversible binding of ellagitannins to salivary or potato proteins (Wang et al., 2013) or to the hydrolysis of punicalin and punicalagin (Cerdá et al. 2003). In the proposed gastro-intestinal hydrolysis pathway, punicalagin breakdown releases equimolar concentrations of ellagic acid and punicalin which is further hydrolyzed to give equimolar concentrations of gallagic acid and glucose (Cerdá et al. 2003).

The loss of punicalagins during the salivary phase of the digestion was not accompanied by the appearance of substantive amounts of ellagic acid; the ellagic acid concentration, in fact, increased after mastication of 31.8 μmol/L, whereas the concentration of punicalagins decreased by about 71.9 μmol/L. This is indicative of the fact that part of the ellagitannins bind potatoes or salivary proteins during the oral phase of the digestion. The concentration of punicalagins remained constant during the gastric phase whereas the intestinal phase caused a further decrease in their concentration. The loss of punicalagins in the intestinal media is totally explained by its hydrolysis to ellagic acid. The punicalagin concentration decreased after intestinal hydrolysis of 71.3 μmol/L which is accompanied by the appearance of 69.3 μmol/L of ellagic acid. It is interesting to note the data of ellagic acid concentration after the gastric phase. The concentration of ellagic acid dropped to 5 μmol/L at the end of the gastric digestion because of its poor solubility in acidic media (Larrosa et al. 2010) and, after the passage in the alkaline intestinal fluid, it returned into the solution. Surprisingly, punicalin concentration did not change further during simulated intestinal digestion respect to the gastric phase. Punicalin was not stable under intestinal conditions but its loss was compensated by the hydrolysis of punicalagin forming punicalin and ellagic acid.

Thus, the increase of ellagic acid that was observed in the last phase of the intestinal digestion is due mostly to the instability of punicalagin in the intestinal environment, with release of ellagic acid moieties and punicalin.

Despite the binding between ellagitannins and proteins and their hydrolysis in the gastro-intestinal media, the pomegranate extract maintained its ability to inhibit starch digestion. This means that hydrolysis of ellagitannins releases compounds with inhibitory activity. For example punicalin and ellagic acid, that are released from punicalagin, are still able to inhibit α-glucosidase and therefore starch hydrolysis during the digestion of potatoes.

There is some *in vivo* and *in vitro* evidence showing that pomegranate juice may be helpful for type II diabetic subjects. Firstly, there are studies reporting the hypoglycemic activity of pomegranate juice in rats (Betanzos-Cabrera et al., 2011) and in diabetic patients (Rock et al. 2008; Rosenblat et al. 2006). Till now the mechanism has not been elucidated, but our data strongly suggest that the hypoglycemic activity of pomegranate juice is due to the ability of ellagitannins to inhibit starch hydrolysis. Some *in vivo* studies highlighted the protective effect of pomegranate juice on some oxidative complications in diabetic patients. Rosenblat et al. (2006) demonstrated that the consumption of pomegranate juice by diabetic patients significantly decreased serum oxidative stress and the extent of oxidized LDL uptake by macrophages. This effect was mediated by PPARγ activation (Shiner et al. 2007). Moreover, the same research group showed that pomegranate juice consumption by diabetic patients could lead to a delay in the atherosclerosis development by increasing paraoxonase 1 stabilization and association with HDL and stimulating its catalytic activity (Betanzos-Cabrera et al. 2011; Fuhrman et al. 2010). This effect is likely mediated by ellagitannins metabolites such as ellagic acid and urolithins (González-Barrio et al. 2010; Park et al. 2011). Pomegranate ellagitannins, in fact, are not absorbed and bioavailable in the human body but are hydrolyzed during the gastro-intestinal digestion releasing ellagic acid that is afterwards bio-transformed in urolithins by the action of colonic microbiota (González-Barrio et al. 2010). Urolithins are well absorbed in the human colon, mainly urolithin-A or urolithin-B and/or iso-urolithin-A according to urolithin phenotype in each person due to the different microbiota communities (Tomás-Barberán et al. 2014), and although they display low antioxidant activity are able *in vitro* to counteract two key features of diabetic complications, i.e. protein glycation and neurodegeneration (Verzelloni et al. 2011). Thus, pomegranate juice (poly)phenols and metabolites could act at different level in attenuates type II diabetic complications. They may act at gastro-intestinal level, where the ellagitannins punicalin, punicalagin and ellagic acid inhibit starch hydrolysis, resulting in a hypoglycaemic effect. At systemic level, the ellagitannins metabolites (ellagic acid, urolithins and their phase II metabolites) may counteract protein glycation and exert anti-atherosclerotic effects, thus reducing some diabetic complications.

5. Conclusions

We were able to identify the ellagitannins punicalin and punicalagin as α-glucosidase inhibitors in pomegranate juice. Ellagitannins retained their inhibitory activity in a *in vitro* model of the digestive system and using cooked potatoes as a source of starch.

In conclusion, our data together with literature data argue with the hypothesis that pomegranate juice can be considered as a rational complementary therapeutic agent to ameliorate postprandial hyperglycaemia linked to type II diabetes and hyperglycaemia-induced vascular complications.

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Figure captions

Figure 1. Dose-dependent inhibition of rat intestinal α-glucosidase activity by pomegranate (poly)phenol-rich extract. The inhibitory activity of the pomegranate extract was measured at concentrations of 150, 300, 400, 600, 1000, 1500, 2000, and 3000 μmol/L. Values represent means of triplicate measurements. Data were analysed with nonlinear regression fit using the log(inhibitor) vs. response model (R^2 = 0.975). Data are means ± SD (*n* = 3).

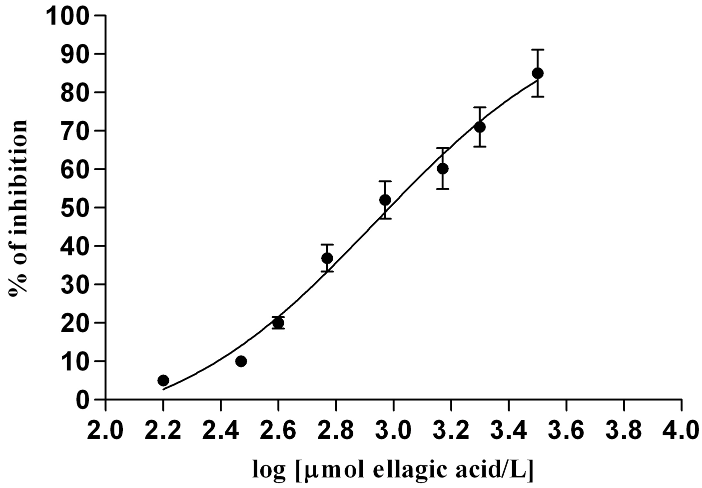
Figure 2. HPLC chromatograms of pomegranate extract anthocyanins (A) and ellagitannins (B) in LH-20 unbound fraction. The monitored wavelength was 520 nm for the detection of anthocyanins and 360 nm for the detection of ellagitannins. Peak numbers as follows: (**1**) delphinidin 3,5-diglucoside, (**2**) cyanidin 3,5-diglucoside, (**3**) pelargonidin 3,5-diglucoside, (**4**) delphinidin 3-glucoside, (**5**) cyanidin 3-glucoside, (**6**) punicalin, (**7**) punicalagin A, (**8**) punicalagin B, (**9**) pedunculagin A, (**10**) granatin B, (**11**) ellagic acid-hex and (**12**) ellagic acid.

Figure 3. HPLC chromatograms of pomegranate extract ellagitannins (A) and anthocyanins (B) in LH-20 bound fraction. The monitored wavelength was 360 nm for the detection of ellagitannins and 520 nm for the detection of anthocyanins. Peak numbers as follows: (**6**) punicalin, (**7**) punicalagin A, (**8**) punicalagin B, (**9**) pedunculagin A, (**10**) granatin B, (**11**) ellagic acid-hex, (**12**) ellagic acid and (**2**) cyanidin 3,5-diglucoside.

Figure 4. (**A**) Effect of changing the order of addition of components on α-glucosidase inhibition by pomegranate extract. In the original assay, the pomegranate extract was mixed with the α-glucosidase and buffer, pre-incubated for 10 min at 37°C and the reaction started by the addition of the substrate. In the revised assay, the pomegranate extract was mixed with the substrate, incubated for 10 min at 37°C and than the reaction was initiated by the addition of the enzyme. The final concentration of pomegranate extract (poly)phenols in the assay was

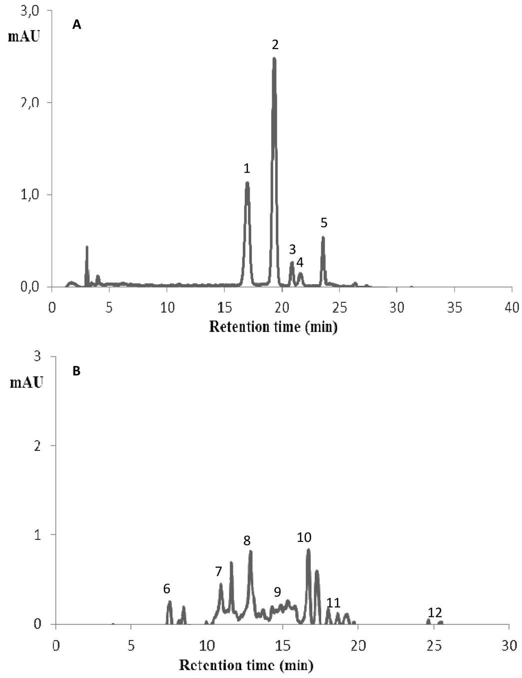
2 mmol/L. Data are means ± SD (n = 3). (**B**) Effect of pre-incubation time and ellagic acid concentration on the α-glucosidase inhibitory activity of ellagic acid. Ellagic acid was pre-incubated for 0, 5, 10, 30, and 60 min with α-glucosidase before the addition of the substrate. Tested ellagic acid concentrations were:( ) 75 µmol/L, (□) 150 µmol/L, (■) 300 µmol/L and (■) 600 µmol/L. * Indicate $P < 0.05$ respect to the previous time. Data are means ± SD (n = 3).

Figure 1



100
90
80
70
60
50
40
30
20
10
0
% of inhibition
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
log [µmol ellagic acid/L]

Figure 2



A
mAU
3,0
2,0
1,0
0,0
1
2
3
4
5
0
5
10
15
20
25
30
35
40
Retention time (min)
B
mAU
3
2
1
0
6
7
8
9
10
11
12
0
5
10
15
20
25
30
Retention time (min)

Figure 3

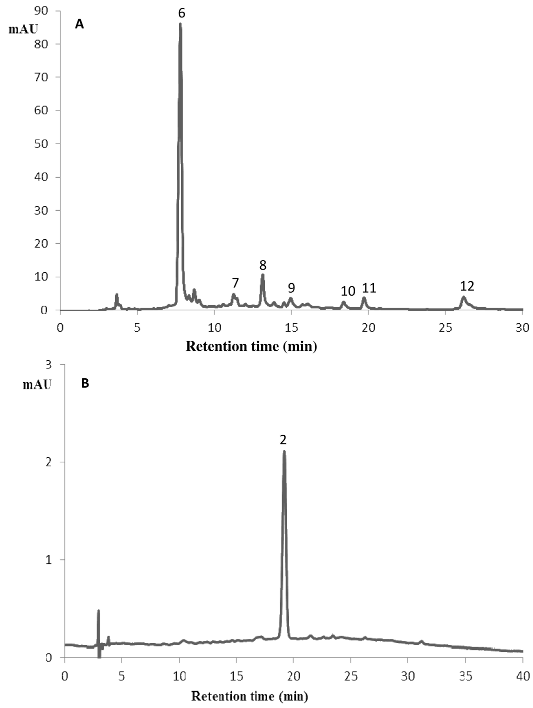
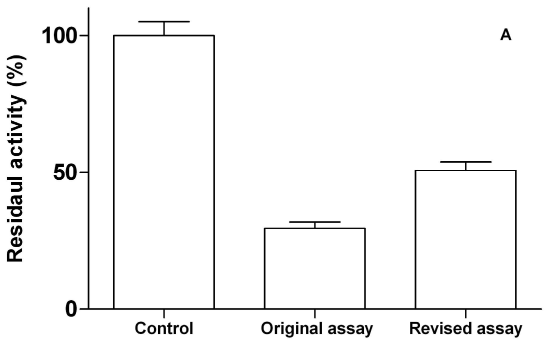

mAU
A
6
7
8
9
10
11
12
Retention time (min)
mAU
B
2
Retention time (min)

Figure 4


A
Residaul activity (%)
100
50
0
Control
Original assay
Revised assay

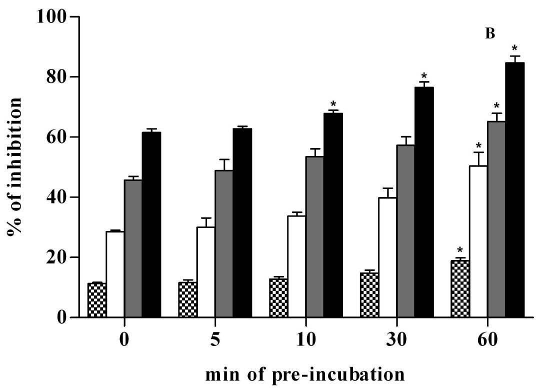

B
% of inhibition
100
80
60
40
20
0
0
5
10
30
60
min of pre-incubation

Table 1

Concentration (μmol/L), retention time and characteristic ions of ellagitannins in pomegranate polyphenol-rich extract

Peak number	Rt	Compound	Concentration [μmol/L]	$[M-H]^-$ m/z	HPLC-ESI(-)-MS/MS *m/z*
6	7.8	Punicalin	652.1 ± 40.5	781	MS^2 [781]: 601, 602, 721
7	11.3	Punicalagin A	85.6 ± 3.2	1083, 541	MS^2 [1083]: 601, 602, 781
8	13.2	Punicalagin B	146.6 ± 7.7	1083, 541	MS^2 [1083]: 601, 602, 781
9	15.0	Pedunculagin A	40.4 ± 1.0	783	MS^2 [783]: 481, 301, 298, 721
10	18.4	Granatin B	19.5 ± 0.3	951	MS^2 [951]: 933, 934, 915, 897
11	19.7	Ellagic acid-hex	31.8 ± 0.3	463	MS^2 [463]: 300, 301, 302
12	26.2	Ellagic acid	74.5 ± 3.6	301	MS^2 [301]: 185, 201, 229
		Total ellagitannins	*1050.5 ± 56.6*		

Data are means ± SD (n = 3).

Table 2

Effects of punicalagin and pomegranate polyphenol-rich extract on V_{max} and K_M values of α-glucosidase.

	Control	Pomegranate polyphenol-rich extract (μmol/L)			Punicalagins (μmol/L)		
		150	300	750	35	70	140
V_{max}	0.050 ± 0.002^{a}	0.047 ± 0.004^{a}	0.045 ± 0.004^{a}	0.035 ± 0.005^{b}	0.036 ± 0.003^{b}	0.033 ± 0.002^{b}	0.030 ± 0.004^{c}
K_M	0.46 ± 0.02^{a}	0.49 ± 0.02^{a}	0.75 ± 0.03^{b}	1.03 ± 0.05^{c}	0.43 ± 0.02^{a}	0.55 ± 0.01^{d}	0.66 ± 0.02^{e}
Inhibition type	/		Mixed			mixed	
K_i (μmol/L)	/		483.80			77.16	

V_{max} is reported as μmol of *p*-nitrophenol per min at pH 6.8 at 37°C whereas K_M is expressed as mmol/L of *p*-nitrophenyl α-D-glucoside.

Data are means ± SD ($n = 3$). Values in the same columns with different lowercase letter are significantly different ($P < 0.05$).

Table 3.

Concentration (µmol/L) of ellagitannins in pomegranate polyphenol-rich extract subjected to *in vitro* gastro-intestinal digestion

	Pomegranate extract (before digestion)	*Post-masticated*	*Post-gastric*	*Post-pancreatic*
Punicalin	652.1 ± 40.5	504.5 ± 60.7*	408.5 ± 40.1	412.3 ± 40.0
Punicalagins	232.2 ± 10.9	160.6 ± 10.1*	147.8 ± 7.6	89.3 ± 10.0*
Ellagic acid	74.5 ± 3.6	106.6 ± 8.6*	5.0 ± 0.3*	175.9 ± 17.6*

* Indicate $P < 0.05$ respect to the previous time. Data are means ± SD ($n = 3$).