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

Andrea Bellesia, Elena Verzelloni, Davide Tagliazucchi*

Department of Life Sciences, University of Modena and Reggio Emilia, Via Amendola, 2 - Pad. Besta, 42100 Reggio Emilia, Italy

* Corresponding author. Tel.: +39-0522-522060; fax: +39-0522-522027

E-mail address: davide.tagliazucchi@unimore.it (D. Tagliazucchi)

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

67 hydrolysis of pomegranate extract was tested against a real food system (potatoes) using an *in*

68 *vitro* digestion model.

69

70 **Methods**

71

72 *Materials*

73

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

86

87 *Sample preparation and total (poly)phenol determination*

88

89 Pomegranate juice (poly)phenol-rich extract was obtained using C18 solid-phase extraction
90 (Verzelloni et al. 2007). Columns were pre-conditioned with 60 mL of methanol and
91 subsequently with 40 mL of 0.1% formic acid in water. The pomegranate juice was loaded
92 (20 mL) and 60 mL of 0.1% formic acid was used to elute unbound materials (free sugars,
93 organic acids and vitamin C). The bound materials, containing pomegranate (poly)phenols,
94 were eluted with 60 ml of methanol. The solvent was removed by a rotary evaporator to near

95 dryness and then freeze-dried. The pomegranate extract was tested for its ability to inhibit the
96 activity of α-glucosidase and α-amylase.
97 Total (poly)phenols in pomegranate juice and extract were determined using the Folin-
98 Ciocalteau method (Singleton et al. 1999). The total phenolic content was expressed in
99 mmol/L of ellagic acid equivalents, using ellagic acid as standard at concentrations ranging
100 between 20 and 1000 mmols/L. The choice of the standard was carried out considering that
101 ellagitannins (which are built up with ellagic acid units) are the most predominant phenolic
102 components present in pomegranate juice (Fischer et al. 2011).
103
104 *Amylase assay*
105
106 Amylase assay was carried out as reported by McDougall et al. (2005) using porcine
107 pancreatic α-amylase and soluble potato starch as a substrate. The reaction was performed in
108 20 mmol/L sodium phosphate buffer pH 6.9 containing 6.7 mmol/L NaCl. For the reaction,
109 0.1 mL of 2 U/mL amylase solution (one unit of amylase is defined as the quantity of enzyme
110 that releases 1.0 mg of maltose from starch in 3 minutes at pH 6.9 at 20°C) was mixed with
111 0.9 mL of sodium posphate buffer or different concentrations of pomegranate extract
112 dissolved in the sodium posphate buffer. After 10 min at 37°C, 1 mL of 1% starch solution
113 (dissolved in the sodium posphate buffer) was added and the reaction mixture was incubated
114 at 37°C for 30 min. The reaction was terminated by adding 1 mL of dinitrosalicylic acid
115 solution and boiling for 15 min in a water bath. Enzyme activity was quantified by measuring
116 the mg of maltose released from starch by reading at 540 nm. To calculate the IC_{50} value, the
117 enzyme activity was determined in the presence of pomegranate extract with phenols
118 concentrations ranging from 150 to 3000 μmol/L. The IC_{50} is defined as the concentration of
119 phenolics required to inhibit 50% of the enzymatic activity.

120

121 *α-Glucosidase assay*

122

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

138

139 *Pomegranate juice (poly)phenol-rich extract fractionation*

140

141 To identify compounds responsible for the inhibitory activity, pomegranate extract was
142 fractionated using Sephadex LH-20 with the method adapted from the *Tannin Handbook*
143 (available at www.users.muohio.edu/hagermae/tannin.pdf). Sorption to Sephadex LH-20 in
144 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.

170 The nebuliser gas temperature was set at 400° C. Helium was used as collision gas at a
171 pressure of $4x10^{-6}$ mbar.
172
173 *HPLC-DAD analysis*
174
175 Individual phenolic compounds were quantified using an HPLC system consisted of a Jasco
176 HPLC system (Orlando FL, U.S.A.) equipped with a diode array detector, a reversed phase
177 column Hamilton HxSil C18 (Hamilton, Reno, Nevada; 250mm x 4.6mm), a volumetric
178 injector Rheodyne (Cotati, CA), and a temperature-controlled oven.
179 For ellagitannins quantification, the monitored wavelength was 360 nm. Identification and
180 quantification of punicalagins A and B, ellagic acid and punicalin in samples were performed
181 using calibration curves of the respective standards compounds. For this reason, a stock
182 solutions of standard compounds were diluted at different concentrations and the solutions
183 were analysed.
184 Anthocyanins were quantified at a wavelength of 520 nm as cyanidin-3-glucoside equivalents.
185 The HPLC parameters were the same as reported in the previous section.
186
187 *Identification of α-glucosidase inhibitors*
188
189 The ellagitannins recognized by LC-MS/MS were tested for their α-glucosidase inhibitory
190 activity. Ellagic acid and punicalagin (a mixture of A and B isomers) were obtained from
191 Sigma Chemical Co. (Milan, Italy) as pure compounds (95% of purity degree). Punicalin was
192 purified from pomegranate juice following the procedure reported in Aviram et al. (2008).
193 Purified compound was evaporated by a rotary evaporator to near dryness and then freeze-
194 dried. The purified compound was characterized by LC-ESI-MS/MS and the purity assayed

195 with HPLC-DAD (95% of purity degree as deduced from the ratio of the peak area of the
196 isolated compounds and total peak area at 280 nm; see supplementary figure).
197 For the calculation of IC_{50} values, α-glucosidase assay was carried out in the presence of
198 variable amounts (from 10 to 500 μmol/L) of punicalin, punicalagin or ellagic acid.
199
200 *In vitro gastro-intestinal digestion*
201
202 The gastro-intestinal system was adapted from Tagliazucchi et al. (2012) with some
203 modifications. Potatoes, selected as real starch-rich food, were weighed, peeled, and cooked
204 whole in boiling water for 30 min. They were removed and cooled at ambient temperature
205 (21°C) to be handled. Ten grams of cooked potatoes (corresponding to 1.71 g of starch) were
206 homogenized in a laboratory blender for 1 min to simulate mastication in presence of 5 mL of
207 simulated salivary fluid and 20 mL of different concentrations of pomegranate extract
208 dissolved in a 0.1 M phosphate-buffer (pH 6.9). The artificial saliva consisted of a 0.1 M
209 phosphate-buffer (pH 6.9) containing 1.336 mmol/L $CaCl_2$, 0.174 mmol/L $MgSO_4$, 12.8
210 mmol/L KH_2PO_4, and 23.8 mmol/L $NaHCO_3$, 2 g/L of food casein (known to be a proline-
211 rich protein), and 150 units/L α-amylase.
212 In the control digestion, the pomegranate extract was omitted and the cooked potatoes (10g)
213 were homogenized in presence of 5 mL of simulated salivary fluid and 20 mL of the 0.1 M
214 phosphate-buffer (pH 6.9).
215 After 10 minutes of incubation at 37°C in a shaking bath, the pH was adjusted to 2.5 (to
216 simulate gastric pH) with concentrated HCl and after 2 g/L of NaCl and 315 U/mL of pepsin
217 were added. The solution was incubated at 37°C in a shaking bath at 100 rpm for 2 h. At the
218 end of the gastric digestion, the pH was brought to 7.5 with $NaHCO_3$ (to simulate hepato-
219 pancreatic pH) before adding 0.8 g/L of pancreatin, 5 mg/mL of bile salts and 2 mL of rat

220 intestinal solution containing 10 U of α-glucosidase. On the basis of the added pancreatin, the
221 amount of digestive enzymes in the intestinal fluid was 80 U/mL of α-amylase, 240 U/mL of
222 proteases and 384 U/mL of lipase. The solution was then incubated at 37°C in a shaking bath
223 at 100 rpm for a further 2 h.
224 The amount of glucose released at the end of the digestion was quantified using a hexokinase,
225 glucose-6-phosphate dehydrogenase, phospho-glucose isomerase method (Kunst et al. 1984).
226 The ellagitannins were quantified by HPLC-DAD as reported in the previous section.
227
228 *Statistical analysis*
229
230 All data are presented as mean ± SD for three replicates for each prepared sample. The
231 Student's t-test and ANOVA with Tukey post-hoc test was performed using Graph Pad Prism
232 (GraphPad Software, San Diego, CA). The differences were considered significant with P
233 <0.05. The IC_{50} values were determined using nonlinear regression analysis and fitting the
234 data with the log(inhibitor) vs. response model (Graph Pad Prism).
235

236 **Results**

237

238 The pomegranate juice contained 6.82 ± 0.75 mmol of ellagic acid equivalent (EAE)/L of
239 phenolic compounds. The percentage of the recovery in the C18 bound fraction,
240 corresponding to the pomegranate extract, was 86% of total (poly)phenols (5.87 ± 0.26 mmol
241 of ellagic acid equivalent (EAE)/L).
242 Pomegranate extract was an effective inhibitor of rat intestinal α-glucosidase with an IC_{50}
243 value of 922.8 ± 1.2 µmol of EAE equivalent/L (**Figure 1**). Acarbose inhibited α-glucosidase
244 in a dose-dependent manner giving an IC_{50} value of 69.7 µmol/L.
245 On the contrary, pomegranate extract was a weak inhibitor of α-amylase. At the highest tested
246 concentration, corresponding to a final concentration of pomegranate (poly)phenols in the
247 assay of 3000 µmol of EAE equivalent/L, the α-amylase activity was inhibited by 42%. These
248 results showed that pomegranate extract contained potent inhibitors of rat intestinal α-
249 glucosidase.
250 The pomegranate extract was fractionated in two different fractions with Sephadex LH-20.
251 The phenolic compounds in the two Sephadex LH-20 fractions were characterised by LC-
252 ESI-MS/MS analysis and the individual compounds quantified by HPLC-DAD analysis. The
253 LH-20 unbound material was pink and contained mainly anthocyanins (**Figure 2**) as
254 delphinidin 3,5-diglucoside (19.6 ± 0.4 µmol/L), cyanidin 3,5-diglucoside (57.5 ± 1.2
255 µmol/L), pelargonidin 3,5-diglucoside (11.6 ± 0.2 µmol/L), delphinidin 3-glucoside (11.1 ±
256 0.2 µmol/L) and cyanidin 3-glucoside (12.8 ± 0.4 µmol/L), low levels of ellagitannins and
257 unidentified flavonols. Instead the LH-20 bound material was brown and contained the
258 majority of ellagitannins (**Figure 3**).
259 Enzymatic analysis showed that only the LH-20 bound fraction caused inhibition of α-
260 glucosidase, whereas the LH-20 unbound fraction did not show any inhibitory activity, even

261 at the highest tested concentrations. It is interesting to note that also the majority of the α-
262 amylase inhibitory activity was recovered in the LH-20 bound fraction, with only a marginal
263 activity found in the LH-20 unbound material.
264 The retention times, concentration (µmol/L) and mass spectral characteristics of the
265 ellagitannins are specified in **Table 1**.
266 Punicalin is the major ellagitannins (peak 6; **figure 3A**) found in the pomegranate extract; this
267 compound present an [M-H]- ion at *m/z* 781 and fragments at *m/z* 601 and 602 for the loss of
268 gallagic acid moiety.
269 Punicalagin showed an [M-H]- ion at *m/z* 1083 but it can be also detected as doubly charged
270 ion species at *m/z* 541. The fragment at *m/z* 601 in MS/MS experiment showed the loss of a
271 gallagic acid moiety and a fragment with *m/z* 781 was observed equivalent to the $[M-H]^-$ ion
272 of punicalin. The presence of the two isomers A and B (peaks 7 and 8; **figure 3A**) was
273 confirmed by the different retention times of the commercial standard isomers.
274 The compound eluting at 15.0 min exhibited an $[M-H]^-$ ion at *m/z* 783. The loss of water
275 moiety and ellagic acid (*m/z* 301) in MS/MS experiment produced fragments at *m/z* 765 and
276 *m/z* 481, respectively. Based on this fragmentation pathway and a previous study (Okuda et
277 al. 1983) this compound was identified as bis-HHDP-hexoside (pedunculagin A; peak 9;
278 **figure 3A**).
279 The compound present in the peak 10 (**figure 3A**) eluted at 18.4 min and exhibited an $[M-H]^-$
280 ion at *m/z* 951. In MS/MS experiment produced fragments at *m/z* 933 and 934. Furthermore,
281 fragments at *m/z* 915 were obtained from the loss of water moiety from principal fragment
282 (m/z 933) and the ion at *m/z* 897 by dehydration. This compound was tentatively identified as
283 granatin B based on the fragmentation pattern reported in previous study (Fischer et al. 2001).
284 The compound which eluted at 19.7 min with fragment at *m/z* 463 was identified as ellagic
285 acid-hexoside (peak 11; **figure 3A**). This compound produced fragments at *m/z* 300, 301, 302

286 in MS/MS experiment, typical m/z fragments of ellagic acid. Ellagic acid-hexoside has
287 previously reported in pomegranate juice and arils (Fischer et al. 2001).
288 The last identified compound was ellagic acid (peak 12; **figure 3A**). The aglycone moiety
289 (*m/z* 301) produced characteristic fragments at *m/z* 229, 201 and 185 in MS/MS experiment.
290 Ellagitannins and ellagic acid were therefore identified as the α-glucosidase inhibitors present
291 in the pomegranate extract. The IC_{50} values of the individual ellagitannins, revealed that
292 punicalagin was the most effective inhibitor of α-glucosidase (IC_{50} of 140.2 ± 1.1 μmol/L)
293 followed by punicalin and ellagic acid (IC_{50} of 191.4 ± 1.3 μmol/L and 380.9 ± 3.5 μmol/L,
294 respectively).
295 To gain more information about the role of each identified ellagitannins in the α-glucosidase
296 activity inhibition, their contribution ratio was calculated by dividing the power of inhibitory
297 activity of each identified compound (calculated by dividing the amount of each single
298 compound in the extract in μmol/L by its IC_{50} value in μmol/L) with that of the pomegranate
299 extract (calculated by dividing the total (poly)phenolic content of the extract in μmol/L by its
300 IC_{50} value in μmol/L) (Toshima et al., 2010). The obtained value was than multiplied by 100
301 to estimate the contribution ratio as %. For example, the contribution ratio of punicalagin was
302 calculated as follows: (232.2/140.2)*100/(5870/922.8) = 26%. The same calculation for
303 punicalin and ellagic acid provides contribution ratio values of 54 and 3%, respectively. The
304 data reported clearly indicated that the α-glucosidase inhibitory activity of pomegranate
305 extract was due to punicalin and punicalagin with a minor contribution of ellagic acid.
306
307 *Kinetic analysis and mechanism of inhibition*
308
309 In the original assay, the pomegranate extract was mixed with α-glucosidase and buffer, pre-
310 incubated for 10 min and the reaction started by the addition of the substrate. If the order of

311 addition of components was changed and the reaction started by the addition of the enzyme
312 rather than the substrate, then the pomegranate extract was less effective (**Figure 4A**). The
313 same effect was observed when different concentrations of ellagic acid were pre-incubated for
314 0, 5, 10, 30 or 60 min with α-glucosidase (**Figure 4B**). This results suggested that
315 pomegranate ellagitannins interacted directly with the α-glucosidase.
316 The ellagitannin punicalagin as well as the pomegranate extract were selected as test
317 inhibitors for the kinetic analysis. All the tested samples reduced the V_{max} and increased K_M
318 of α-glucosidase (**Table 2**). These results suggested a mixed-type inhibition with respect to
319 substrate concentration.
320
321 *Effect of pomegranate extract on potato starch hydrolysis during in vitro gastro-intestinal*
322 *digestion*
323
324 The ability of the pomegranate extract to inhibit starch hydrolysis was assessed using a real
325 food during simulated gastro-intestinal conditions. Cooked potatoes was firstly subjected to
326 mastication, in presence of simulated salivary fluid which contained 150 units/L of α-amylase.
327 After 10 minutes, the bolus was subjected to consecutive gastric (2 h) and intestinal (2 h)
328 digestion, in presence of 80 units/mL of α-amylase and 370 units/L of α-glucosidase.
329 At the end of the gastro-intestinal digestion, in absence of the pomegranate extract, the
330 amount of released glucose was 199.5 ± 2.12 mg/g of potato starch. The addition of the
331 pomegranate extract in the digestive system produced a decrease in the amount of released
332 glucose at the end of the gastro-intestinal digestion of 18 and 44% when the digestion was
333 carried out with 2.35 or 4.7 mmol/L of total (poly)phenols, respectively. Control experiments
334 carried out without enzymes showed that there was no hydrolysis of potato starch.

335 The behaviour of the ellagitannins during simulated gastro-intestinal digestion of potatoes
336 was followed with HPLC-DAD. The results are detailed in **Table 3**. The concentration of the
337 ellagitannins punicalin and punicalagin decreased by 22.6 and 30.9% after mastication and by
338 36.8 and 61.6% after pancreatic digestion, respectively. The amount of ellagic acid increases
339 to 142.8 and 234.2% after mastication and pancreatic digestion, respectively.
340

341 **Discussion**

342

343 This is the first report showing that pomegranate juice (poly)phenolic extract is a potent
344 inhibitor of *in vitro* carbohydrate digestion. Pomegranate extract strongly inhibited the rat
345 intestinal α-glucosidase activity *in vitro*.
346 The ability of the pomegranate (poly)phenolic-rich extract to inhibit the starch hydrolysis was
347 also demonstrated using an *in vitro* digestion system, mimicking the physiological gastro-
348 intestinal condition, and potatoes as food rich in starch.
349 A variety of food (poly)phenolic extracts have been shown to inhibit α-amylase and α-
350 glucosidase activities *in vitro*. Rat intestinal α-glucosidase inhibitory activity of pomegranate
351 extract (IC_{50} value of 278 μg/mL) is lower than that of acarbose (IC_{50} of 45 μg/mL),
352 anthocyanins-rich berry extracts (such as blueberry, blackcurrant, rowanberry, and
353 strawberry; IC_{50} values from 18 to 42 μg/mL), and black tea (IC_{50} of 64 μg/mL) (McDougall
354 et al., 2005, Koh et al. 2010). However, the *in vitro* inhibitory activity of pomegranate extract
355 was similar to that of green tea (IC_{50} value of 297 μg/mL) which has been found to be
356 effective in reducing postprandial blood glucose level *in vivo* (Tang et al. 2013).
357 The inhibitory activity against both the enzymes was assigned to ellagitannins, especially
358 punicalin and punicalagin. The comparison of the IC_{50} values against rat intestinal α-
359 glucosidase of punicalagin and punicalin (140.2 and 191.4 μmol/L, respectively) with that of
360 other (poly)phenols revealed that these compounds are effective as theaflavin digallate (IC_{50}
361 of 165 μmol/L, Koh et al. 2010) and diacilated anthocyanins (IC_{50} of 200 μmol/L, Matsui et
362 al. 2002). Pomegranate ellagitannins are more effective than green tea catechins (Koh et al.
363 2010), and flavonols (Tadera et al. 2006). Pomegranate ellagitannins are less effective than
364 acarbose (IC_{50} of 69.7 μmol/L).

365 Punicalagin, despite its lower IC_{50} value against rat intestinal α-glucosidase, was not the most
366 important contributor to the inhibitory activity (26% of contribution). In contrast, punicalin
367 was estimated to be the main contributor to pomegranate extract α-glucosidase inhibition
368 (54% of contribution) owing to its higher content in the extract. The total contribution ratio of
369 all identified ellagitannins in this study was 83%, suggesting that some unidentified
370 compounds with α-glucosidase inhibitory activity can be present in the pomegranate extract or
371 that synergic effects should be considered.
372 Kinetic analysis suggested that pomegranate extract, and ellagitannins inhibited α-glucosidase
373 activity in a mixed mode. The pre-incubation and the order of addition experiments indicate
374 that ellagitannins influence α-glucosidase activity via their ability to bind proteins (Wang et
375 al. 2013). The non-specific binding of ellagitannins with α-glucosidase may alter the structure
376 of the enzyme by reducing the velocity of the catalysis and the accessibility to the active site
377 of the substrate.
378 Most of the studies previously published on the inhibitory activity of (poly)phenols or
379 (poly)phenols-rich extract against α-amylase and α-glucosidase were carried out using
380 enzymatic assay that did not represent the physiological conditions of the gastro-intestinal
381 tract. One of the most important criticisms in employing the enzymatic assay is the use of
382 starch solution or synthetic substrate solution instead of real food. The importance of utilizing
383 real food lies in the presence of additional molecules (such as proteins, lipids and fibers),
384 other than starch, that may impede the effect of (poly)phenols on the enzymes. An additional
385 criticism is related to the fact that phenolic compounds are somewhat unstable under real or
386 simulated gastro-intestinal conditions. For example it has been shown that anthocyanins are
387 degraded in the pancreatic media (Liu et al. 2014) whereas ellagitannins may undergo partial
388 breakdown in the gastro-intestinal tract (Larrosa et al. 2010). To overcome this point, we
389 tested the ability of pomegranate (poly)phenols to inhibit the carbohydrate hydrolysis during

390 simulated digestion of potatoes. Results show that the pomegranate extract is able to inhibit in
391 a concentration dependent manner potato starch digestion under *in vitro* gastro-intestinal
392 conditions. Despite all the limitations of the model system (static model, glucosidase not
393 bound to the enterocyte membrane), our results allow us to infer that pomegranate
394 (poly)phenols may be effective inhibitors of starch digestion also *in vivo* by inhibiting the
395 activity of α-glucosidase. Our results show that a portion of pomegranate juice (200 ml) is
396 able to inhibit the starch hydrolysis by about 50% during the digestion of a portion (100 g) of
397 potatoes. As already observed, ellagitannins are not stable under gastro-intestinal condition
398 (Larrosa et al. 2010). We found a decrease in the concentration of the ellagitannins punicalin
399 and punicalagin by 22.6 and 30.9% after mastication. These decreases may be due to the
400 irreversible binding of ellagitannins to salivary or potato proteins (Wang et al., 2013) or to the
401 hydrolysis of punicalin and punicalagin (Cerdá et al. 2003). In the proposed gastro-intestinal
402 hydrolysis pathway, punicalagin breakdown releases equimolar concentrations of ellagic acid
403 and punicalin which is further hydrolyzed to give equimolar concentrations of gallagic acid
404 and glucose (Cerdá et al. 2003).
405 The loss of punicalagins during the salivary phase of the digestion was not accompanied by
406 the appearance of substantive amounts of ellagic acid; the ellagic acid concentration, in fact,
407 increased after mastication of 31.8 μmol/L, whereas the concentration of punicalagins
408 decreased by about 71.9 μmol/L. This is indicative of the fact that part of the ellagitannins
409 bind potatoes or salivary proteins during the oral phase of the digestion. The concentration of
410 punicalagins remained constant during the gastric phase whereas the intestinal phase caused a
411 further decrease in their concentration. The loss of punicalagins in the intestinal media is
412 totally explained by its hydrolysis to ellagic acid. The punicalagin concentration decreased
413 after intestinal hydrolysis of 71.3 μmol/L which is accompanied by the appearance of 69.3
414 μmol/L of ellagic acid. It is interesting to note the data of ellagic acid concentration after the

415 gastric phase. The concentration of ellagic acid dropped to 5 μmol/L at the end of the gastric
416 digestion because of its poor solubility in acidic media (Larrosa et al. 2010) and, after the
417 passage in the alkaline intestinal fluid, it returned into the solution. Surprisingly, punicalin
418 concentration did not change further during simulated intestinal digestion respect to the
419 gastric phase. Punicalin was not stable under intestinal conditions but its loss was
420 compensated by the hydrolysis of punicalagin forming punicalin and ellagic acid.
421 Thus, the increase of ellagic acid that was observed in the last phase of the intestinal digestion
422 is due mostly to the instability of punicalagin in the intestinal environment, with release of
423 ellagic acid moieties and punicalin.
424 Despite the binding between ellagitannins and proteins and their hydrolysis in the gastro-
425 intestinal media, the pomegranate extract maintained its ability to inhibit starch digestion.
426 This means that hydrolysis of ellagitannins releases compounds with inhibitory activity. For
427 example punicalin and ellagic acid, that are released from punicalagin, are still able to inhibit
428 α-glucosidase and therefore starch hydrolysis during the digestion of potatoes.
429 There is some *in vivo* and *in vitro* evidence showing that pomegranate juice may be helpful
430 for type II diabetic subjects. Firstly, there are studies reporting the hypoglycemic activity of
431 pomegranate juice in rats (Betanzos-Cabrera et al., 2011) and in diabetic patients (Rock et al.
432 2008; Rosenblat et al. 2006). Till now the mechanism has not been elucidated, but our data
433 strongly suggest that the hypoglycemic activity of pomegranate juice is due to the ability of
434 ellagitannins to inhibit starch hydrolysis. Some *in vivo* studies highlighted the protective
435 effect of pomegranate juice on some oxidative complications in diabetic patients. Rosenblat et
436 al. (2006) demonstrated that the consumption of pomegranate juice by diabetic patients
437 significantly decreased serum oxidative stress and the extent of oxidized LDL uptake by
438 macrophages. This effect was mediated by PPARγ activation (Shiner et al. 2007). Moreover,
439 the same research group showed that pomegranate juice consumption by diabetic patients

440 could lead to a delay in the atherosclerosis development by increasing paraoxonase 1
441 stabilization and association with HDL and stimulating its catalytic activity (Betanzos-
442 Cabrera et al. 2011; Fuhrman et al. 2010). This effect is likely mediated by ellagitannins
443 metabolites such as ellagic acid and urolithins (González-Barrio et al. 2010; Park et al. 2011).
444 Pomegranate ellagitannins, in fact, are not absorbed and bioavailable in the human body but
445 are hydrolyzed during the gastro-intestinal digestion releasing ellagic acid that is afterwards
446 bio-transformed in urolithins by the action of colonic microbiota (González-Barrio et al.
447 2010). Urolithins are well absorbed in the human colon, mainly urolithin-A or urolithin-B
448 and/or iso-urolithin-A according to urolithin phenotype in each person due to the different
449 microbiota communities (Tomás-Barberán et al. 2014), and although they display low
450 antioxidant activity are able *in vitro* to counteract two key features of diabetic complications,
451 i.e. protein glycation and neurodegeneration (Verzelloni et al. 2011). Thus, pomegranate juice
452 (poly)phenols and metabolites could act at different level in attenuates type II diabetic
453 complications. They may act at gastro-intestinal level, where the ellagitannins punicalin,
454 punicalagin and ellagic acid inhibit starch hydrolysis, resulting in a hypoglycaemic effect. At
455 systemic level, the ellagitannins metabolites (ellagic acid, urolithins and their phase II
456 metabolites) may counteract protein glycation and exert anti-atherosclerotic effects, thus
457 reducing some diabetic complications.
458

459 **5. Conclusions**

460

461 We were able to identify the ellagitannins punicalin and punicalagin as α-glucosidase
462 inhibitors in pomegranate juice. Ellagitannins retained their inhibitory activity in a *in vitro*
463 model of the digestive system and using cooked potatoes as a source of starch.
464 In conclusion, our data together with literature data argue with the hypothesis that
465 pomegranate juice can be considered as a rational complementary therapeutic agent to
466 ameliorate postprandial hyperglycaemia linked to type II diabetes and hyperglycaemia-
467 induced vascular complications.

References

Adhami VM, Khan N, Mukhtar H (2009). Cancer chemoprevention by pomegranate: laboratory and clinical evidence. Nutrition and Cancer 61:811–815.

Aviram M, Volkova N, Coleman R, Dreher M, Kesava R M, Ferreira D, Rosenblat M (2008). Pomegranate phenolics from the peels, arils, and flowers are antiatherogenic: studies in vivo in atherosclerotic apolipoprotein E-deficient (E^0) mice and in vitro in cultured macrophages and lipoproteins. Journal of Agricultural and Food Chemistry 56:1148–1157.

Betanzos-Cabrera G, Guerrero-Solano JA, Martínez-Pérez MM, Calderón-Ramos Z G, Belefant-Miller H, Cancino-Diaz JC (2011). Pomegranate juice increases levels of paraoxanase1 (PON1) expression and enzymatic activity in streptozotocin-induced diabetic mice fed with a high-fat diet. Food Research International 44:1381–1385.

Boath AS, Grussu D, Stewart D, McDougall G J (2012a). Berry polyphenols inhibit digestive enzymes: a source of potential health benefits? Food Digestion 3:1–7.Boeing H, Bechthold A, Bub A, Ellinger S, Haller D, Kroke A, Leschik-Bonnet E, Müller MJ, Oberritter H, Schulze M, Stehle P, Watzl B (2012). Critical review: vegetables and fruit in the prevention of chronic diseases. European Journal of Nutrition 51:637–663.

Cerdá B, Llorach R, Cerón JJ, Espín JC, Tomás-Barberán FA (2003). Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice. European Journal of Nutrition 42:18–28.

Del Rio D, Rodriguez-Mateos A, Spencer JPE, Tognolini M, Borges G, Crozier A (2013). Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxidants & Redox Signaling 18:1818−1892.

Di Silvestro RA, Di Silvestro DJ, Di Silvestro DJ (2009). Pomegranate extract mouth rinsing effects on saliva measures relevant to gingivitis risk. Phytotherapy Research 23:1123−1127.

Dolara P, Luceri C, De Filippo C, Femia AP, Giovannelli L, Caderni G, Cecchini C, Silvi S, Orpianesi C, Cresci A (2005). Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. Mutation Research 591:237–246.

Fischer UA, Carle R, Kammerer DR (2011). Identification and quantification of phenolic compounds from pomegranate (Punica granatum L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD–ESI/MS^n. Food Chemistry 127:807–821.

Fuhrman B, Volkova N, Aviram M (2010). Pomegranate juice polyphenols increase recombinant paraoxonase-1 binding to high-density lipoprotein: Studies in vitro and in diabetic patients. Nutrition 26:359-366.

González-Barrio R, Borges G, Mullen W, Crozier A (2010). Bioavailability of anthocyanins and ellagitannins following consumption of raspberries by healthy humans and subjects with an ileostomy. Journal of Agricultural and Food Chemistry 58:3933–3939.

Halliwell B, Zhao K, Whiteman M (2000). The gastrointestinal tract: a major site for antioxidant action? Free Radical Research 33:819–830.

Kaur M, Singh RP, Gu M, Agarwal R, Agarwal C (2006). Grape seed extract inhibits *in vitro* and *in vivo* growth of human colorectal carcinoma cells. Clinical Cancer Research 12:6194–6201.

Koh L W, Wong LL, Loo YY, Kasapis S, Huang D (2010). Evaluation of different teas against starch digestibility by mammalian glycosidases. Journal of Agricultural and Food Chemistry 58:148–154.

Krentz AJ, Bailey CJ (2005). Oral antidiabetic agents. Current role in type 2 diabetes mellitus. Drugs 65:385–411.

Kunst A, Draeger B, Ziegenhorn J (1984). Methods of Enzymatic Analysis 3rd Edition, Academic Press, Vol. 6, 163-172.

Kwon YI, Apostolidis E, Shetty K (2008). Inhibitory potential of wine and tea against α-amylase and α-glucosidase for management of hyperglycemia linked to type 2 diabetes. Journal of Food Biochemistry 32:15–31.

Larrosa M, García-Conesa MT, Espín JC, Tomás-Barberán FA (2010). Ellagitannins, ellagic acid and vascular health. Molecular Aspects of Medicine 31:513–539.

Li Y, Wen S, Kota BP, Peng G, Li GQ, Yamahara J, Ruofogalis BD (2005). Punica granatum flower extract, a potent alpha-glucosidase inhibitor, improves postprandial hyperglycemia in Zucker diabetic fatty rats. Journal of Ethnopharmacology 99:239–244.

Liu Y, Zhang D, Wu Y, Wang D, Wei Y, Wu J, Ji B (2014). Stability and absorption of anthocyanins from blueberries subjected to a simulated digestion process. International Journal of Food Science and Nutrition 65:440–448.

Matsui T, Ebuchi S, Kobayashi M, Fukui K, Sugita K, Terahara N, Matsumoto K (2002). Anti-hyperglycemic effect of diacylated anthocyanin derived from *Ipomoea batatas* cultivar Ayamurasaki can be achieved through the α-glucosidase inhibitory action. Journal of Agricultural and Food Chemistry 50:7244–7248.

McDougall GJ, Stewart D (2005). The inhibitory effects of berry polyphenols on digestive enzymes. BioFactors 23:189-195.

McDougall GJ, Shpiro F, Dobson P, Smith P, Blake A, Stewart D (2005). Different polyphenolic components of soft fruits inhibit α -amylase and α-glucosidase. Journal of Agricultural and Food Chemistry 53:2760-2766.

Medjakovic S, Jungbauer A (2013). Pomegranate: a fruit that ameliorates metabolic syndrome. Food & Function 4:19–34.

Nathanson D, Nyström T (2009). Hypoglycemic pharmacological treatment of type 2 diabetes: targeting the endothelium. Molecular and Cellular Endocrinology 297:112–126.

Oki T, Matsui T, Osajima Y (1999). Inhibitory effect of alpha-glucosidase inhibitor varies according to its origin. Journal of Agricultural and Food Chemistry 47:550–553.

Okuda T, Yoshida T, Ashida M, Yazaki K (1983). Tannins of *Casuarina* and *Stachyurus* species. Part 1. Structures of pedunculagin, casuarictin, strictinin, casuarinin, casuariin and stachyurin. Journal of the Chemical Society, Perkin Transactions, 1, 1765-1772.

Park SH, Kim JL, Lee ES, Han SY, Gong JH, Kang MK, Kang YH (2011). Dietary ellagic acid attenuates oxidized LDL uptake and stimulates cholesterol efflux in murine macrophages. Journal of Nutrition 141:1931–1937.

Rock W, Rosenblat M, Miller-Lotan R, Levy AP, Elias M, Aviram M (2008). Consumption of wonderful variety pomegranate juice and extract by diabetic patients increases paraoxonase 1 association with high-density lipoprotein and stimulates its catalytic activities. Journal of Agricultural and Food Chemistry 56:8704–8713.

Rosenblat M, Hayek T, Aviram M (2006). Anti-oxidative effects of pomegranate juice (PJ) consumption by diabetic patients on serum and on macrophages. Atherosclerosis 187:363–371.

Shiner M, Fuhrman B, Aviram M (2007). Macrophage paraoxonase 2 (PON2) expression is up-regulated by pomegranate juice phenolic anti-oxidants via PPAR gamma and AP-1 pathway activation. Atherosclerosis 195:313–321.

Singleton VL, Orthofer R, Lamuela-Raventos RM (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology 99:152–178.

Tadera K, Minami Y, Takamatsu K, Matsuoka T (2006). Inhibition of alpha-glucosidase and alpha amylase by flavonoids. Journal of Nutritional Science and Vitaminology 52:149–153.

Tagliazucchi D, Verzelloni E, Conte A (2012). The first tract of alimentary canal as an extractor. Release of phytochemicals from solid food matrices during simulated digestion. Journal of Food Biochemistry 36:555-568.

Tang W, Li S, Liu Y, Huang MT, Ho CT (2013). Anti-diabetic activity of chemically profiled green tea and black tea extracts in a type 2 diabetes mice model via different mechanisms. Journal of Functional Foods 5:1784–1793.

Tomás-Barberán FA, García-Villalba R, González-Sarrías A, Selma MV, Espín JC (2014). Ellagic acid metabolism by human gut microbiota: consistent observation of three urolithin phenotypes in intervention trials, independent of food source, age, and health status. Journal of Agricultural and Food Chemistry 62: 6535–6538.

Toshima A, Matsui T, Noguchi M, Qiu J, Tamaya K, Miyata Y (2010). Identification of α-glucosidase inhibitors from a new fermented tea obtained by tea-rolling processing of loquat (*Eriobotrya japonica*) and green tea leaves. Journal of the Science of Food and Agriculture 90:1545–1550.

Usta C, Ozdemir S, Schiariti M, Puddu PE (2013). The pharmacological use of ellagic acid-rich pomegranate fruit. International Journal of Food Science and Nutrition 64:907–913.

Verzelloni E, Tagliazucchi D, Conte A (2007). Relationship between the antioxidant properties and the phenolic and flavonoid content in traditional balsamic vinegar. Food Chemistry 105:564–571.

Verzelloni E, Pellacani C, Tagliazucchi D, Tagliaferri S, Calani L, Costa LG, Brighenti F, Borges G, Crozier A, Conte A, Del Rio D (2011). Antiglycative and neuroprotective activity of colon-derived polyphenol catabolites. Molecular Nutrition and Food Research 55:35–43.

Wang Y, Zhang H, Liang H, Yuan Q (2013). Purification, antioxidant activity and protein-precipitating capacity of punicalin from pomegranate husk. Food Chemistry 138:437–443.

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

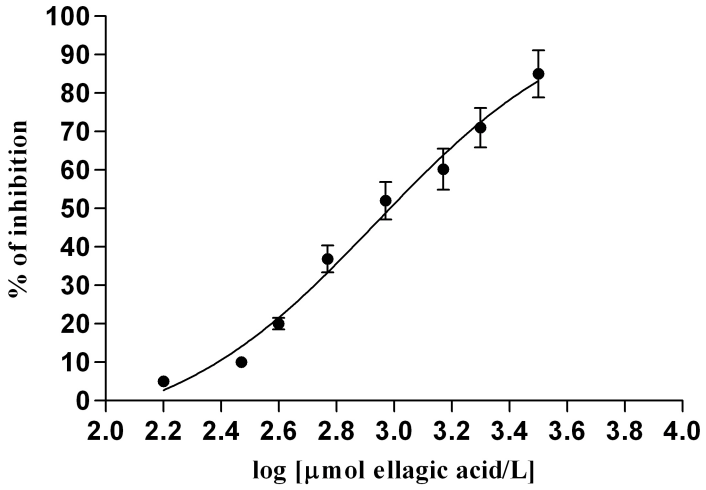

100
90
80
70
60
50
40
30
20
10
0
% of inhibition
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
log [µmol ellagic acid/L]

Figure 2

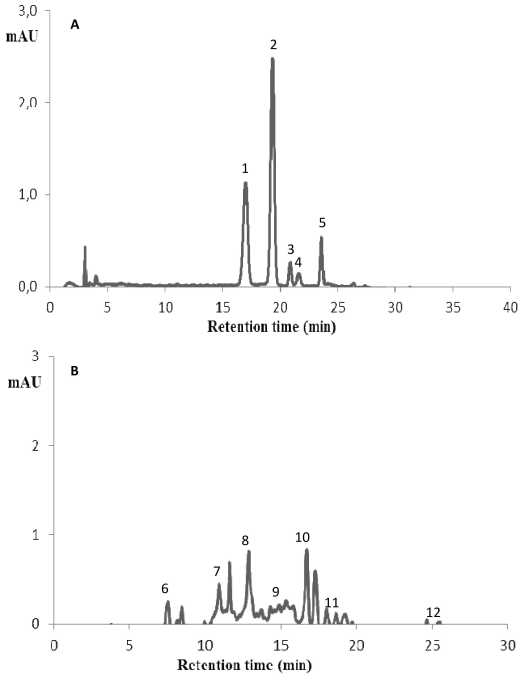

A
mAU
3,0
2,0
1,0
0,0
1
2
3
4
5
0
5
10
15
20
25
30
35
40
Retention time (min)
B
mAU
3
2
1
0
6
7
8
9
10
11
12
0
5
10
15
20
25
30
Retention time (min)

Figure 3

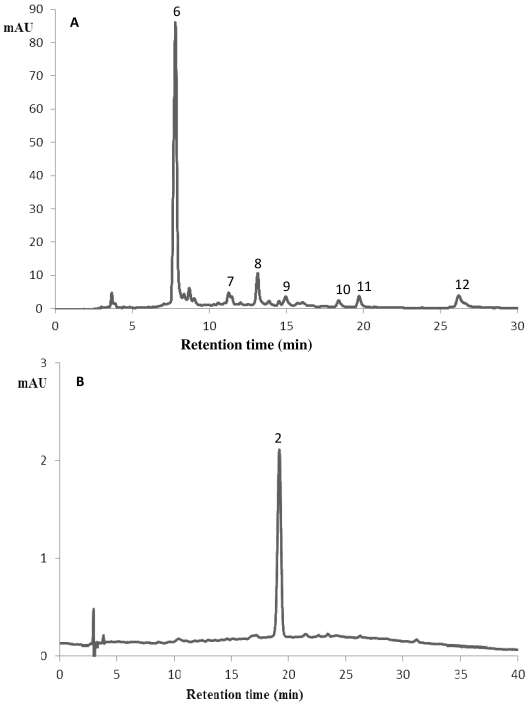
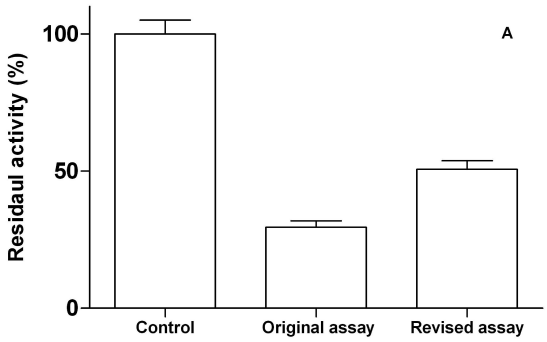

A
mAU
90
80
70
60
50
40
30
20
10
0
6
7
8
9
10
11
12
0
5
10
15
20
25
30
Retention time (min)
B
mAU
3
2
1
0
2
0
5
10
15
20
25
30
35
40
Retention time (min)

Figure 4


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

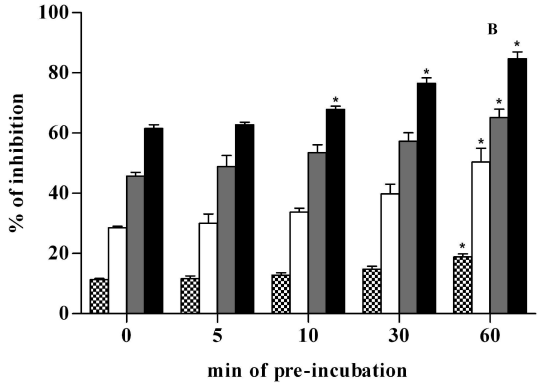

B
% of inhibition
100
80
60
40
20
0
0
5
10
30
60
min of pre-incubation

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 ($n = 3$).