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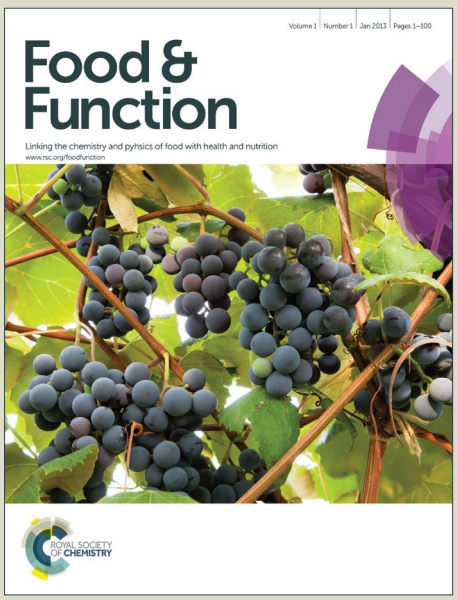

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1 # *In vitro* bioaccessibility and functional properties of polyphenols from pomegranate peels and
2 # pomegranate peels-enriched cookies

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Abstract

Obesity is an urgent social problem and new functional foods providing polyphenols and dietary fibers (DF) may be promising tools to modulate oxidative stress, inflammation and energy homeostasis.

Pomegranate peels (PPe) are an agro-industrial by-product containing polyphenols such as ellagitannins (ETs), gallic acid (GA), ellagic acid (EA) and its derivatives (EAs), as well as DF.

In this study, PPe enriched cookies (PPeC) were developed and the bioaccessibility of their polyphenols as well as the ability to exert antioxidant activity along the gastro-intestinal tract (GiT) and to modulate digestive enzymes was evaluated *in vitro*.

Data showed that the potential bioaccessibility of ETs was 40% lower from PPeC than PPe whereas EAs and GA bioaccessibility increased by 93% and 52% for PPeC compared to PPe. The concentration of the polyphenols at each digestion step was associated with the total antioxidant capacity of the potentially bioaccessible material. Moreover the polyphenols released in the simulated duodenal phase upon PPeC digestion exhibited inhibitory activity towards α-glucosidase, α-amylase and lipase, being for α-glucosidase > α-amylase > lipase.

In conclusion data demonstrated that the inclusion of PPe at 7.5% in a bakery product potentially leaded to a high bioaccessibility of ETs degradation products (mainly EA and EAs) in the duodenum, with a consequent antioxidant protection along the GiT and modulation of glucose metabolism. Further human studies are warranted to evaluate whether these effects also occur *in vivo*.

Introduction

Obesity consists in excessive body fat accumulation resulting from an imbalance between individual energy intake and expenditure. In 2014, more than 1.9 billion of adults were overweight and over 600 million of these were obese.[1] The tremendous increase of obesity worldwide is associated to an increase among population of non communicable chronic diseases (NCCD), such as cardiovascular diseases, type 2 diabetes, and some types of cancer.[2,3] Subclinical oxidative stress and inflammation are key factors in the pathogenesis of obesity-related diseases.[4] Healthy lifestyle, including energy balanced and variegate dietary regimens, together with a sufficient level of physical activity, are known to be the best strategies to fight overweight and associated diseases.[1] However, educational campaign and promotion of physical activity failed among population. Thus, the consumption of foods providing bioactive compounds, such as polyphenols and dietary fibers (DF), may represent an effective strategy to prevent obesity and the risk of associated NCCD.[5-8]

Jointly with DF, polyphenols are very promising ingredients for the formulation of functional foods aiming to target gastro-intestinal tract (GiT) and metabolic pathways underpinning appetite and body weight control.[8-13] In fact, beside their ability to quench free radicals forming in the GiT, thus acting as antioxidants, polyphenols may influence the activity of digestive enzymes, such as α-amylase, α-glucosidase and pancreatic lipase, thus modulating nutrients bioavailability and the hormonal response triggered by foods.[4,14-20]

The chemical structure of polyphenols, as well as their disposition in the food matrix can highly influence their availability to react against free radicals and to inhibit digestive enzymes in the GiT. Therefore, the study of bioaccessibility of polyphenols along the GiT, also using *in vitro* human digestion models, is very useful to foresee their functional effects in human body.

Fruits, vegetables and whole grains are the most important dietary sources of polyphenols.[21,22]

Nevertheless, by-products of plant food processing may represent a natural and cheap promising source of polyphenols for nutraceutical purpose.[23] They can be extracted to recover purified biophenols or, more advantageously, they may be used as whole powder and added as functional ingredients in food formulation, providing also a considerable amount of DF.[24,25]

In this frame, pomegranate peels (PPe) represent a very promising agro-industrial by-product, due to their high content of bioactive compounds such as ellagitannins (ETs), mainly including punicalagin, gallic acid (GA), ellagic acid (EA) and its derivatives (EAs), as well as DF (33-62% of PPe on dry weight).[26-28] The pomegranate fruits are majorly processed by food industry to obtain the juice from the arils, while PPe, representing about 50% of fresh fruit weight, are discarded and are commercially available as a dietary supplement.[29] PPe have higher content of total polyphenols and higher antioxidant capacity than the pulp fraction.[30] Several evidence indicate that ETs and EA from pomegranate may be protective against many chronic diseases, such as some types of cancers, type 2 diabetes, atherosclerosis and cardiovascular diseases.[31,32] Recent *in vitro* studies pointed out that the benefits of ETs and EA from pomegranate was due to their anti-inflammatory, antioxidant and digestive enzyme inhibitory action along the GiT.[15,31,33-40]

Only few studies proposed PPe powder as functional ingredient to increase the antioxidant capacity, the nutritional value and the shelf-life of some foods, such as sunflower oil, wheat bread, cookies and meat products.[41-46] A very recent *in vitro* study showed that a PPe flour could release ETs and EA during an *in vitro* sequential enzyme digestion, might modify antioxidant status and lead to the production of short-chain fatty acids inside the GiT.[47] However, a lack of knowledge still exists in the literature regarding the potential functionality of PPe when used as functional ingredient in baked food products.

This study aimed at assessing the bioaccessibility of polyphenols from a PPe powder and PPe-enriched cookies (PPeC), their potential antioxidant capacity along the GiT as well as the ability of PPeC to modulate glucose and lipid metabolism. To this purpose, PPeC were developed and *in vitro* sequential enzyme digestions coupled to LC/MS/MS and biochemical analyses of the fractions

collected at each digestion steps were used to determine the chemical transformations induced by baking process on PPe polyphenols and to assess the step-by-step release of antioxidant activity as well as the overall potential antioxidant capacity of PPeC.

Materials and Methods

Chemicals

Water and methanol used for the analysis were of HPLC grade (Merck, Germany). Ethanol and formic acid were purchased from VWR international (Fontenay-sous-Bois, France). Cellulose powder was obtained from Fluka (Buchs, Switzerland). Total dietary fiber assay kit was purchased from Megazyme International (Wicklow, Ireland). Calcium chloride, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl, 95% (DPPH), sodium bicarbonate, hydrochloric acid, acetic acid, celite, 5,5′-dithiobis(2-nitrobenzoic acid), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 2,3-dimercapto-1-propanol tributyrate, starch Azure, 4-morpholinoethanesulfonic acid (MES), para-nitrophenyl-α-d-glucopyranoside, sodium phosphate dibasic heptahydrate were purchased from Sigma-Aldrich (St. Louis, MO). Standards of punicalagin, EA and GA were purchased from Sigma-Aldrich (St. Louis, MO). Lipase from *Aspergillus niger* (187 U/g), α-amylase from porcine pancreas (type VI-B ≥ 10 U/mg), α-glucosidase from *Saccharomyces cerevisiae* (Type I ≥ 10 U/mg), bacterial protease from *Streptomyces griseus* (Pronase E, Type XIV ≥ 3.5 U/mg), cell wall degrading enzyme complex from *Aspergillus* sp. (Viscozyme L), pancreatin from porcine pancreas (4 X USP) and pepsin from porcine gastric mucosa (≥ 250 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO). The ingredients for cookies preparation were purchased from a local market.

PPe powder dietary fiber

A commercially available PPe powder was used in this study. It was purchased from Detrade UG (Bremen, Germany). The content of total dietary fiber (TDF), including soluble dietary fiber (SDF) and insoluble dietary fiber (IDF), was determined using the protocol described by Prosky *et al*..[48]

PPeC preparation

PPeC and control cookies (CTC), were prepared according to a recipe described in AACC (American Association of Cereal Chemists), method 10-54 (AACC, 2000) with some modifications.[49] Particularly, dough was prepared mixing 40 g of wheat flour, 5 g of PPe, 17.5 g of sugar, 10 g of butter, 0.8 g of $NaHCO_3$, 0.2 g of NaCl and 18 g of water. In CTC, PPe powder was substituted with the same amount of inert cellulose powder. To achieve maximum homogeneity between the two samples, each dough was rolled between two bars with a height of 3 mm and was shaped in a disk of 30 mm diameter. CTC and PPeC were baked simultaneously at 170°C for 12 min in a forced-air circulation oven. The final concentration of PPe powder in PPeC was 7.5%.

In-vitro sequential enzyme digestion of PPe powder and PPeC

The sequential enzyme digestions of PPe, PPeC and CTC were performed following the method described by Papillo *et al*., slightly modified.[50] The scheme of the adopted experimental protocol is reported in **Figure 1**.

Briefly, four sequential digestive phases, namely simulated salivary phase (SSP), gastric phase (SGP), duodenal phase (SDP) and colon phase (SCP), including Viscozyme L and Pronase E steps, were carried out.

PPe (2.5 g), grinded PPeC (2.5 g), cellulose (2.5 g, as control for PPe) and grinded CTC (2.5 g) were submitted to the enzymatic digestion. At each step, the supernatants were collected for the HPLC analysis and for the measure of the total antioxidant capacity (TAC), whereas the pellets

were analyzed for the evaluation of insoluble TAC. Before the analysis, the supernatants were further centrifuged (4000 rpm for 30 min at 4°C), ultra-centrifuged (14800 rpm for 15 min at 4°C) and different aliquots (one milliliter each) of digestive extracts were carefully dried under nitrogen flow, in order to preserve the samples.

HPLC analysis

For the HPLC analysis of soluble fractions, the aliquots of digestive extracts were re-suspended in 1 mL of a solution water/methanol (50:50, v/v) at 0.5 % of formic acid and properly diluted.

The chromatographic separation of ETs, EA, EAs and GA from the supernatants at each digestion step was obtained according the methods described by Seeram *et al*., with slight modifications.[51,52] The HPLC system consisted in two binary pumps (LC-10AD, Shimadzu, Kyoto, Japan), equipped with a photo-diode array detector (SPD-M10A, Shimadzu, Kyoto, Japan) and a C-18 reversed phase column Gemini 5µ 250 mm x 4.6 (Phenomenex, Torrance, CA) was used. The flow rate was 0.8 mL/min and the mobile phases were water at 2% of formic acid (phase A) and methanol (phase B); the following gradient was applied: [min]/[%B] - (0/0), (20/10), (30/30), (35/40), (40/70), (45/98), (48/98), (50/0), (52/0). The injection volume was 20 µl.

ETs, EA, EAs and GA were identified by LC-MS/MS with an API 3000 Triple Quadrupole mass spectrometer (Applied Biosystem Sciex). Mass spectrometry conditions were optimized, according to Fischer *et al.*.[26] ETs, including punicalin (anomers α and β), punicalagin (anomers α and β), pedunculagin (anomers α and β) and a compound previously identified by Fischer *et al*., as a castalagin derivative, were detected and quantified at 378 nm. For EA, EAs and GA, the wavelengths used were 366 nm and 280 nm, respectively. Calibration curves of punicalagin, EA and GA were obtained. Punicalagin, EA and GA were identified and quantified with the corresponding standards. Punicalin anomers, pedunculagin anomers, castalagin derivative and total ETs were quantified as punicalagin equivalents. EAs, including ellagic acid hexoside, pentoside and deoxyhexoside, were quantified as EA equivalents.

Total antioxidant capacity of soluble and insoluble fractions

The total antioxidant capacity (TAC) of soluble and insoluble fractions obtained by each digestive step, was measured with a spectrophotometer (PG Instruments, UK) set at 517 nm, using respectively the DPPH method and DPPH QUENCHER method, as previously described by Papillo *et al.*.[50]

Before the analysis, the dried aliquots of the soluble fractions were re-suspended in 1 mL of water and the insoluble pellets obtained after each digestion step were freeze dried. The results were expressed as mmol of Trolox equivalents (TE)/kg of dry matter (DM).

α-glucosidase inhibition assay

The assay was carried out as reported by Chandran *et al.*, with slight modifications.[53] The aliquots of extract from SDP, previously dried under nitrogen flow, were re-suspended in distilled water and appropriately diluted. Subsequently, 125 µL of this solution were added to 870 µL of 0.1 M phosphate buffer (pH 6.8). Then, 125 µL of a 0.1 M phosphate buffer solution (pH 6.8) containing the enzyme α-glucosidase from *Saccharomyces cerevisiae* (1 U/mL) were added. The reaction was started adding to the mixture, 125 µL of a 0.1 M phosphate buffer solution containing para-nitrophenyl-α-d-glucopyranoside (3 mM) as substrate for the enzyme. The samples were incubated for 20 min at 37°C, in a shaking thermostatic bath. The amount of released p-nitrophenol was determined spectrophotometrically, measuring the absorbance of the solution at 405 nm. Percent inhibition was calculated as follows:

$$\frac{[(\text{Abs } 100\% - \text{Abs } 0\%) - (\text{Abs sample} - \text{Abs blank})]}{(\text{Abs } 100\% - \text{Abs } 0\%)} \times 100$$

where: Abs 100% was the absorbance of 100 % enzyme activity (in the reaction mixture only the enzyme and the substrate); Abs 0% was the absorbance of 0% enzyme activity (in the reaction mixture only the substrate without enzyme); Abs sample was the absorbance of the reaction

mixture, containing substrate, enzyme and the tested extract; Abs blank was the absorbance of the reaction mixture without enzyme and containing only the substrate and tested extract.

α-amylase inhibition assay

The capacity of SDP to inhibit the activity of α-amylase was investigated as reported by Dey *et al.*, with slight modifications.[54]

The dried aliquots of SDP extracts were reconstituted in 1 mL of distilled water and properly diluted. The starch Azure was used as substrate. Particularly, 4 mg of starch Azure were suspended in 0.4 mL of a 0.1 M Tris-HCl buffer (pH 6.9), containing calcium chloride (0.01 M) and the solution was boiled for 5 minutes and pre-incubated 5 minutes at 37 °C. Then, 0.4 mL of extract diluted in 0.1 M Tris–HCl buffer (pH 6.9) were added to the substrate. Finally, the reaction was started adding 0.2 mL of a 0.1 M Tris-HCl buffer solution (pH 6.9), containing α-amylase from porcine pancreas (2 U/mL). The reaction mixture was incubated 10 minutes at 37 °C, in a shaking thermostatic bath. The reaction was stopped by adding 1 mL of 50% (v/v) acetic acid solution. Afterwards, the samples were ultra-centrifuged at 14800 rpm for 10 min. at 4°C. The absorbance of supernatant was measured at 595 nm with a spectrophotometer (PG Instruments, UK). Percent inhibition was calculated, as follows:

$$\frac{[(\text{Abs } 100\% - \text{Abs } 0\%) - (\text{Abs sample} - \text{Abs blank})]}{(\text{Abs } 100\% - \text{Abs } 0\%)} \times 100$$

where: Abs 100% was the absorbance of 100 % enzyme activity (in the reaction mixture only the enzyme and the substrate); Abs 0% was the absorbance of 0% enzyme activity (in the reaction mixture only substrate without enzyme); Abs sample was the absorbance of the reaction mixture, containing the substrate, enzyme and the tested extract; Abs blank was the absorbance of the reaction mixture without the enzyme and containing only the substrate and tested extract.

187 ***Lipase inhibition assay***

188 Lipase inhibition assay was carried out with a method adapted by Mennella *et. al.*.[55]
189 Preliminarily, one milliliter of SDP extract, previously dried under nitrogen flow, was re-suspended
190 in one milliliter of distilled water and appropriately diluted. One milliliter of 0.1 M Tris–HCl buffer
191 solution (pH 8.3), containing 0.3 mM 5-5′-dithio-bis (2-nitrobenzoic acid) (DTNB), was mixed with
192 50 µl of 0.1 M Tris–HCl buffer solution (pH 8.3) containing the enzyme (0.05 U/mL) and 40 µl of
193 the solution of intestinal extract. Then, 80 µL of 20 mM 2,3-dimercapto-1-propanol tributyrate
194 (dissolved in ethanol) were added in order to start the reaction. The samples were incubated in a
195 shaking thermostatic bath for 30 min at 37°C. The lipase released 2,3-dimercapto-1-propanol that
196 reacted with DTNB to release TNB anions (yellow colour). Tubes were centrifuged at 14800 rpm
197 for 10 min at 4°C and the absorbance was measured at 412 nm with a spectrophotometer (PG
198 Instruments, UK). Percent inhibition was calculated as follows:

$$\frac{[(\text{Abs } 100\% - \text{Abs } 0\%) - (\text{Abs sample} - \text{Abs blank})]}{(\text{Abs } 100\% - \text{Abs } 0\%)} \times 100$$

199 where: Abs 100% was the absorbance of 100 % enzyme activity (in the reaction mixture only the
200 enzyme and the substrate); Abs 0% was the absorbance of 0% enzyme activity (in the reaction
201 mixture only substrate without enzyme); Abs sample was the absorbance of the reaction mixture
202 containing substrate, enzyme and the tested extract; Abs blank was the absorbance of the reaction
203 mixture without enzyme and containing only the substrate and tested extract.

204 ***Statistical analysis***

205 According to Papillo *et al.,*[50] the TAC of all the soluble fractions plus the TAC of the last insoluble
206 residue were used to calculate the overall potential TAC (OP-TAC), of which the FREE-TAC was
207 related to the SSP, while the BOUND-TAC was due to the soluble fractions collected in all the
208 steps plus the last residue. In addition, the total amount of ETs, EAs and GA released at all

digestion steps were used to calculate the overall bioaccessible ETs (OB-ETs), EAs (OB-EAs) and GA (OB-GA), respectively. The amount of each class of compounds released in the SSP were the FREE-ETs, FREE-EAs and FREE-GA, while the amount released after SSP (from stomach to colon) were the BOUND-ETs, BOUND-EAs and BOUND-GA.

The sequential enzymatic digestion was performed in triplicate and all the samples were always analysed in triplicate. The data were analysed by ANOVA, and the mean values were compared by Tukey test ($\alpha = 0.05$), using XLStat statistical software (Addinsoft, New York, NY). The Pearson correlation coefficient was used for the bivariate correlation analysis between variables ($\alpha = 0.05$).

Results

PPe dietary fiber

TDF content of PPe powder was 48 g/100g of PPe, whose 72.9% was IDF and 27.1% was SDF.

Bioaccessibility of polyphenols from PPe and from PPeC

Punicalin anomers, punicalagin anomers, pedunculagin anomers, castalagin der., GA , EA and three EAs were identified and quantified in the extracts of each step of simulated *in vitro* digestion of PPe and PPeC whereas they were absent in cellulose and CTC. The results were summarized in **Table 1.**

The total amount of bioaccessible ETs, EAs and GA from PPe were 7666.3 mg/100g of PPe powder, of which the 42.8% (3278.7 mg/100g of PPe powder) were immediately released in the SSP, the 52.0% were potentially bioaccessible in the upper GiT and the 5.2% were potentially bioaccessible only after in the SCP.

The total amount of bioaccessible ETs, EAs and GA from PPeC was 491.1 mg/100g of PPeC, of which 223.2 mg (45.4%) were from the salivary phase, thus being potentially bioaccessible early in the proximal GiT, while the remaining part 267.9 mg (54.6%) were released in the next steps. In particular, 216.8 mg/100g of PPeC (44.1% of the total polyphenols released) were potentially bioaccessible in the upper GiT (stomach and duodenum) after the action of pepsin and pancreatin,

while 51.1 mg (10.4% of the total polyphenols released) were potentially bioaccessible in the lower GiT (colon) after Pronase E and Viscozyme L action. The extract from SSP was mainly constituted by both punicalagin and EA, whereas the subsequent extracts were mainly constituted by EA.

In **Table 2**, the values of overall bioaccessible (OB)-polyphenols (i.e. OB-ETs, OB-EAs, OB-GA), as well as the percentage of free-polyphenols (i.e. FREE-ETs, FREE-EA, FREE-GA) and bound-polyphenols (i.e. BOUND-ETs, BOUND-EAs and BOUND-GA) for both PPeC and for PPe were reported. Data showed that despite huge differences of repartition between free and bound fractions of specific polyphenols out of the overall bioaccessible amount from PPe and PPeC, similar percentages for total polyphenols were found.

Total antioxidant capacity

Figure 2 reported the TAC of soluble and insoluble fractions after each step of the *in vitro* digestion of both PPe that PPeC.

Data obtained by PPe digestion showed that the TAC of fractions solubilized gradually decreased along the GiT. The TAC of insoluble fraction (pellet) after SCP (colon) was higher than that obtained after SGP, thus indicating that a great amount of TAC was released after the disruption of the DF matrix in PPe.

Similarly, during PPeC digestion, the TAC of the fraction solubilized in the SSP was always higher than the others but a higher TAC of the SDP than SGP extract was observed. The insoluble TAC found for PPeC followed the same trend along the digestive steps as for PPe, except the TAC of SCP pellet that was only a bit higher than that relative to SDP.

The FREE-TAC and BOUND-TAC were evaluated both for PPe and for PPeC. For PPe, the values of FREE-TAC and BOUND-TAC were 3307 mmol TE/kg of DM (29% of OP-TAC) and 7933 mmol TE/kg of DM (71% of OP-TAC), respectively. BOUND-TAC calculated for the extracts after the SGP, SDP and SCP were 26%, 23% and 16% of the total OP-TAC, respectively.

Likewise, for PPeC, FREE-TAC and BOUND-TAC were 162 mmol TE/kg of DM (31% of OP-TAC) and 360 mmol TE/kg of DM (69% of OP-TAC), respectively. The values of BOUND-TAC calculated for the extracts after the SGP, SDP and SCP were 21%, 25% and 17% of the total OP-TAC, respectively.

The OP-TAC calculated for PPe and PPeC were 11240 mmol TE/kg of DM and 522 mmol TE/kg of DM, respectively. In **Figure 3**, the values of OP-TAC calculated for PPE and PPeC were compared with the values of OP-TAC, calculated for five common plant foods.

Inhibition of enzyme activity

SDP extracts from PPeC digestion were tested in order to assay their capacity to inhibit *in vitro* α-amylase, α-glucosidase, and lipase. The results were reported in **Figure 4**. Data demonstrated that SDP strongly inhibited α-glucosidase activity, while a weak inhibitory capacity against α-amylase and lipase was found. This effect was due to the whole mixture of polyphenols (ETs, EA, EAs and GA) present in SDP extract. It was calculated that 0.007 mg of polyphenols in SDP extract inhibited by 84% the activity of 1 U of α-glucosidase, whereas about 5 (0.036 mg) and 80 (0.578 mg) greater amounts of the same mixture of polyphenols were necessary to inhibit by 72% and by 13% 1 U of α-amylase and 1 U of lipase, respectively.

Discussion

For the first time in this study the potential TAC and bioaccessibility of polyphenols from a PPe powder and a PPe-enriched baked food, as well as their ability to inhibit the activity of α-amylase, α-glucosidase and lipase, were evaluated upon an *in vitro* simulated gastrointestinal digestion.

PPeC developed in this study contained 7.5% of PPe, providing 491.1 mg/100g of PPeC of overall bioaccessible polyphenols (mainly ETs and EA), and 3.6 % of DF according to Hasnaoui *et al.* and Viuda-Martos *et al.*.[28,56] This formulation guaranteed sensory acceptability of the product according to Ismail *et al.*[41] and Srivastava *et al.*[57] and allowed the nutritional claim of *"source of dietary fiber"*

281 according to European Commission Regulation 1924/2006 and European Food Safety Authority
282 (EFSA).[58,59]
283 Previous *in vivo* study demonstrated that under physiological conditions ETs are partially
284 hydrolyzed and release EA. EA is then gradually metabolized in the intestine to produce bioactive
285 urolithin D, urolithin C and finally urolithin A and urolithin B in the distal parts of the intestine.[31]
286 Interestingly, our data showed that the potential bioaccessibility of ETs was 40% lower while that
287 of EAs and GA was respectively 93% and 52% higher from PPeC than PPe.
288 In particular, comparing the release of polyphenols obtained from PPeC upon the *in vitro* digestion
289 with the expected values extrapolated from the data obtained for PPe, and taking into account the
290 enrichment of PPe in PPeC (7.5% by weight), data showed (**Table 1**) a 13.4% reduced overall
291 release of polyphenols from PPeC compared to the expected concentration. This total difference
292 was due to a halved release of ETs, and a doubled and a 1.5 fold increased release of EAs and GA
293 from PPeC compared to the expected values, respectively.

294 It is likely that during the process to produce cookies, ETs (mainly punicalin and punicalagin) and
295 EA present in PPe, partially hydrolyzed by forming EA and GA, respectively, in PPeC.[60]
296 Moreover, it was hypothesized that some of ETs, such as castalagin der., could become more
297 bioaccessible from PPe under cookies processing because a double amount (122.4 mg/100g of
298 PPeC vs 62.2 mg/100g of PPeC) was retrieved in the solubilized fractions after *in vitro* digestion
299 compared to the expected amount. Interestingly, looking at the step by step release of castalagin der.
300 upon the simulated gastrointestinal digestion, data showed that the 63.1% of the increased release
301 occurred in the SSP whereas the 31.9% and the 4.8% were released in the SDP and in the SCP,
302 respectively. On the other hand, the newly formed GA in PPeC was completely released in the SSP
303 (140%) while the newly formed EAs were released by 66% and by 32% in the SDP and SCP
304 respectively and only the 14% was present in the SSP.

These data demonstrated that the complex food matrix of PPeC, mainly including starch and the gluten network together with PPe, strongly influenced the release of EAs, mainly EA, so that the enzyme action of pepsin and pancreatin was necessary to allow their delivery from the food matrix. This happened because polyphenols can interact with food macronutrients through covalent and non-covalent interactions, the resultant affinity being also influenced by the water solubility of polyphenols: higher is the water-solubility lower is the affinity with non-polar food matrices.[61-64] EA is much less soluble in water than ETs and GA, thus potentially forming stronger hydrophobic interactions with the water insoluble gluten matrix of PPeC, and being mostly released after the disruption of that matrix compared to its more water-soluble glycosylated derivatives (mainly detected in the SSP).[29,31] In other words, data showed that the use of PPe as ingredient in the cookies determined a different bioaccessibility of the constituent polyphenols compared to the PPe powder, thus influencing the related potential antioxidant protection. In fact, a significant correlation among the total amount of ETs, EAs and GA, released at each digestive step, and the values of TAC measured for the corresponding soluble extracts, both for PPe ($r = 0.983$; $p = 0.017$) and for PPeC ($r = 0.969$; $p = 0.031$) was observed. Moreover, the effect of digestive enzymes on the two types of matrix (PPe and PPeC) caused a different behavior of the un-solubilized materials at each digestive step. In particular, results showed that, insoluble fractions of PPe and PPeC after the SCP, i.e. after the action of bacterial proteases and cellulolytic enzyme mixture, exhibited a higher antioxidant activity than the insoluble material obtained by the SDP.

The potential antioxidant protection in the gut demonstrated *in vitro* in this study might be at the basis of the reduced colon inflammation and positive modulation of the gut microbiota induced by PPe in obese mice fed with a high-fat diet.[65]

In PPe, like in cereals, fruits and vegetables, polyphenols are found free or covalently bound to cell wall structural components, such as arabinoxylans, cellulose and lignin, or to proteins.[66] So, once ingested the action of digestive enzymes allows the polyphenols to be delivered by the food matrix and act against reactive oxygen species (ROS), along the GiT.[12,13,50] On the other hand the

destruction of the matrix operated by the enzymes allows the bound polyphenols to dispose on the surface of the un-solubilized material and to act as a sponge of free radicals along the GiT before arriving to the colon and be delivered by the gut microbiota enzymes.[13]

Mounting evidence indicate that the interactions among the gut microbiota, DF and polyphenols may have implications on the prevention of obesity risk. The potential antioxidant activity along the GiT, demonstrated in this study by PPe and PPeC, is perfectly in line with the conclusion driven by Al-Muammar and Khan, that the prevention of obesity risk, associated with consumption of pomegranate products, is correlated to their antioxidant compounds released along the GiT.[38]

Data of the present study showed that values of FREE-TAC were 29% and 31% of OP-TAC, for PPe and PPeC, respectively. These results were similar to those obtained for apple (32%) and spinach (29%) in a previous study from our research group, and a bit lower than those relative to walnuts (42%).[50] However, PPe exhibited an OP-TAC that was about ten times higher than the OP-TAC calculated for the walnuts (see **Figure 3**). This was in line with the huge abundance of the whole mix of antioxidants (ETs, EA, EAs and GA) in pomegranate by-products (85 g/kg of d.w.) compared to walnuts (1.8 to 3.0 g for kg of fresh weight), particularly to the higher relative abundance of hydrolysable tannins in pomegranate by-products (about 99.5%) than in walnuts kernels (about 76.3%).[26,67] In fact, compared to other polyphenols, hydrolysable tannins are recognized highly antioxidants and very active inhibitors of free radicals. These effects were directly correlated to their molecular weight and to the number of reactive galloyl groups in the molecules.[68] Punicalagin is recognized as one of strongest antioxidant compound and major responsible of the high antioxidant capacity of pomegranate juice being 16 folds more antioxidant than EA and 7 folds more antioxidant than GA.[26,27,69]

Together with the antioxidant potential, our data also indicated that PPeC could influence glucose metabolism and lipid digestion by modulating activity of α-glucosidase, α-amylase and lipase.

Starch is the most common complex carbohydrate in foods, and its digestion in the GiT is mainly mediated by α-amylase and α-glucosidase. The rate of glucose release in the GiT modulate energy homeostasis and fine physiological mechanisms underlying hunger and satiety.[70]

The findings of the present study indicated that the soluble extract from SDP strongly inhibited α-glucosidase and, at a lesser extent, α-amylase.

The results on α-glucosidase inhibition were consistent with those previously obtained by Li *et al*..[39] with PPe phenolics and those obtained by Çam *et al.*[71] with a functional ice cream containing PPe phenolics-enriched microcapsules.

The use of α-glucosidase from *Saccharomyces cerevisiae* in the present study could be considered as a limitation because it is known that the extent of activity of α-glucosidase inhibitors may vary according to the origin of α-glucosidase, i.e. if they are α-glucosidase from yeast or from mammalians.[72-75] However, in previous studies polyphenols from PPe extracts mainly including punicalagin, punicalin and ellagic acid strongly inhibited *in vitro* rat intestinal α-glucosidase and recombinant human maltase–glucoamylase but exhibited weak or no inhibitory effects against porcine pancreatic α-amylase.[34,36,76] So, it might be speculated that the results found in the present study using a pure yeast α-glucosidase might be found also using a mammalian enzyme, but future studies are warranted to demonstrate this hypothesis.

On the other hand, lipase is the major enzyme responsible for the gastrointestinal digestion of dietary triglycerides into monoglycerides and free fatty acids. The inhibition of pancreatic lipase along the GiT could result in a reduced lipid absorption and in a consequent reduced energy intake. The weak inhibitory effect of SDP extract on lipase activity was in line with results previously obtained *in vitro* and with the *in vivo* evidence that consumption of a pomegranate leaf extract could inhibit the development of obesity and hyperlipidemia in high-fat diet induced obese mice also through inhibition of the pancreatic lipase activity.[15,40]

Conclusion

In conclusion, the findings of the present study support the use of PPe at a dose of 7.5% by weight in bakery food products, as a functional ingredient to counteract oxidative stress along the GiT. The potential antioxidant protection may involve all the organs of the GiT due to the release of ETs, EA, EAs and GA along all the digestive system. Moreover it was demonstrated that the baking process increased the amount of EA in the PPeC due to chemical degradation of ETs, thus allowing its increased release in the duodenal and colon phase compared to the raw ingredient. In fact, food matrix strongly influenced both the release of polyphenols in the different steps as well as the antioxidant potential of the un-solubilized material. Together with a high antioxidant potential, the mix of polyphenols solubilized in the SDP also acted as strong inhibitors of α-glucosidase thus possibly influencing in a positive manner the glucose metabolism *in vivo*.

I*n vivo* studies are warranted to evaluate the sensory properties of the PPeC developed in this study and its effects on oxidative stress and on glucose and lipid metabolism.

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The authors declare no competing financial interest.

Conflicts of interest

There are no conflicts of interest to declare.

Abbreviations

GiT: Gastrointestinal tract

PPe: Pomegranate peels

PPeC: Pomegranate peels-enriched cookies

ETs: Ellagitannins

EA: Ellagic acid

EAs: Ellagic acid derivatives

GA: Gallic acid

SDP: Simulated duodenal phase

DF: Dietary fiber

CTC: Control cookies

SSP: Simulated salivary phase

SGP: Simulated gastric phase

SCP: Simulated colon phase

TAC: Total antioxidant capacity

TE: Trolox equivalents

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Table 1: Concentrations (mg/100g) of polyphenols released upon enzymatic digestion of PPe and PPeC. For PPeC the percentage variation (Var.) of each concentration vs the expected concentration was also reported. Expected concentrations were calculated on the basis of data obtained by PPe *in vitro* digestion, taking into account the enrichment of PPe in PPeC (7.5% by weight). Values were expressed as mean ± SD. For each compound, values in the same row followed by different lower case letter (for PPe digestion) or different upper case letter (for PPeC digestion) are significantly different ($p < 0.05$) according to Tukey's test.

[#] mg of punicalagin equiv. + mg of EA equiv. + mg of GA equiv. HE: Hexoside; PE: Pentoside; DHE: Deoxyhexoside; < LOD: Lower than the limit of detection; n.a.: not available.

Compounds	Simulated salivary phase			Simulated gastric phase			Simulated duodenal phase			Simulated colon phase			Total		
	PPe	PPeC	Var.	PPe	PPeC	Var.	PPe	PPeC	Var.	PPe	PPeC	Var.	PPe	PPeC	Var.
	mg/100g	mg/100g	(%)	mg/100g	mg/100g	(%)	mg/100g	mg/100g	(%)	mg/100g	mg/100g	(%)	mg/100g	mg/100g	(%)
Punicalin	855.9±193.0 ^{a}	33.1±1.4 ^{A}	-48	924.1±60.9 ^{a}	11.8±2.5 ^{B}	-83	240.1±17.9 ^{b}	4.3±0.1 ^{C}	-76	62.3±2.9 ^{b}	< LOD	n.a.	2082.4±166.3	49.2± 1.2	-68
Punicalagin	1491.6±122.4 ^{a}	57.0±8.0 ^{A}	-48	1039.8±62.1 ^{b}	22.9±1.4 ^{B}	-70	426.6±54.9 ^{c}	14.9±3.2 ^{B}	-53	74.1±16.7 ^{d}	< LOD	n.a.	3032.1±218.2	94.8±9.8	-58
Pedunculagin	62.1±14.4 ^{a}	2.4±0.4 ^{A}	-48	23.9±3.1 ^{b}	1.1±0.5 ^{B}	-39	15.3±3.1 ^{b,c}	< LOD	n.a.	0.7±0.1 ^{c}	< LOD	n.a.	102.0±18.4	3.5±0.3	-53
Castalagin der.	73.3±6.3 ^{c}	43.4±6.5 ^{B}	704	15.9±2.3 ^{d}	1.5±0.4 ^{D}	25	569.2±18.4 ^{a}	61.3±2.7 ^{A}	46	181.7±7.1 ^{b}	16.3±0.8 ^{C}	22	840.1±19.8	122.4± 8.7	97
ETs	2482.9±70.9 ^{a}	135.9±16.0 ^{A}	-26	2003.7±114.1 ^{b}	37.2±4.0 ^{C}	-75	1251.2±78.5 ^{c}	80.5±5.9 ^{B}	-13	318.8±20.7 ^{d}	16.3±0.8 ^{D}	-31	6056.6±156.9	270.1±17.2	-40
Ellagic acid HE	88.2±4.6 ^{a}	3.8±0.7 ^{A}	-42	41.4±0.9 ^{b}	2.1±0.2 ^{B}	-32	20.2±0.2 ^{c}	1.9±0.1 ^{B}	27	5.3±0.3 ^{d}	0.6±0.0 ^{C}	50	155.1± 5.2	8.4±0.4	-27
Ellagic acid PE	23.3±0.4 ^{a}	1.6±0.3 ^{A}	-6	< LOD	0.3±0.1 ^{C}	n.a.	8.0±0.1 ^{b}	1.0±0.1 ^{B}	67	2.8±0.1 ^{c}	0.3±0.0 ^{C}	50	34.1±0.3	3.2±0.4	28
Ellagic acid DHE	29.6±2.8 ^{a}	1.7±0.1 ^{A}	-23	12.3±3.6 ^{b}	0.7±0.1 ^{C}	-22	9.9±1.8 ^{b}	1.0±0.1 ^{B}	43	2.6±0.1 ^{c}	0.2±0.0 ^{D}	0	54.4±8.2	3.6± 0.1	-10
Ellagic acid	537.5±12.3 ^{a}	55.9±5.3 ^{B}	40	341.4±71.3 ^{b}	14.6±0.6 ^{D}	-42	136.8±1.1 ^{c}	69.1±0.3 ^{A}	584	59.6±5.2 ^{d}	33.5±0.7 ^{C}	661	1075.2±86.4	173.1±4.0	117
EAs	678.6±13.7 ^{a}	63.0±6.4 ^{B}	25	395.1±68.5 ^{b}	17.7±0.8 ^{D}	-39	174.9±3.0 ^{c}	73.1±0.3 ^{A}	467	70.3±4.9 ^{d}	34.6±0.8 ^{C}	565	1318.8±83.0	188.4±4.6	93
Gallic acid	117.3±14.2 ^{a}	24.3±1.9 ^{A}	179	103.2±1.7 ^{a}	7.5±1.2 ^{B}	-1	57.5±1.2 ^{b}	0.8±0.2 ^{C}	-81	12.9±0.1 ^{c}	< LOD	n.a.	291.0±16.5	32.6±0.9	52
ETs + EAs + GA [#]	3278.7±90.0 ^{a}	223.2±24.3 ^{A}	-8	2502.0±77.7 ^{b}	62.5±6.1 ^{C}	-66	1483.6±80.0 ^{c}	154.3±6.0 ^{B}	41	402.0±25.5 ^{d}	51.1±1.5 ^{C}	72	7666.3±122.9	491.1±22.7	-13

645 **Table 2:** Overall bioaccessible-ETs (OB-ETs), Overall bioaccessible-EAs (OB-EAs) and Overall bioaccessible-GA
646 (OB-GA), for PPe and PPeC,. FREE-ETs, FREE-EAs and FREE-GA corresponded to the amount released in the SSP
647 while the amount released later on the digestion were the BOUND-ETs, BOUND-EAs and BOUND-GA.

	Compounds	Overall bioaccessible (mg/100g)	Free (mg/100g)	Free (%)	Bound (mg/100g)	Bound (%)
PPe	ETs	6056.6 ± 156.9	2482.9 ± 70.9	41	3573.5 ± 169.4	59
	EAs	1318.8 ± 83.0	678.6 ± 13.7	51	640.3 ± 70.4	49
	GA	291.0 ± 16.5	117.3 ± 14.2	40	173.7 ± 2.7	60
	Total	7666.3 ± 122.9	3278.7 ± 90.0	43	4387.6 ± 116.7	57
PPeC	ETs	270.1 ± 17.2	135.9 ± 16.0	50	134.2 ± 1.6	50
	EAs	188.4 ± 4.6	63.0 ± 6.4	33	125.4 ± 1.8	67
	GA	32.6 ± 0.9	24.3 ± 1.9	75	8.3 ± 1.0	25
	Total	491.1 ± 22.7	223.2 ± 24.3	45	267.9 ± 2.4	55

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651 **Figure captions**

652 **Figure 1:** Flow chart of the experimental design. Enzyme inhibition assays were performed only for
653 PPeC.

654 **Figure 2**: TAC of soluble and insoluble fractions from simulated gastrointestinal digestion of PPe
655 and PPeC. Mean values were expressed as mmol of TE/kg of DM. Both for PPe and for PPeC
656 digestion steps, different lower case letter (for soluble TAC) and upper case letter (for insoluble
657 TAC) indicate significantly different values ($p < 0.05$) according to Tukey's test.

658 **Figure 3:** Comparison among OP-TAC values of PPe and PPeC and OP-TAC values of 5 common
659 plant foods as reported by Papillo *et al.*[50]. Values were expressed as mmol of TE/kg of DM.

660 **Figure 4:** Enzyme inhibition rate of extracts from SDP of PPeC. The dilution factor of the tested
661 extracts, compared with the initial concentration, was 30, 6 and 6, for α-glucosidase, α-amylase and
662 lipase, respectively. Data were corrected for the inhibition elicited by CTC.
663

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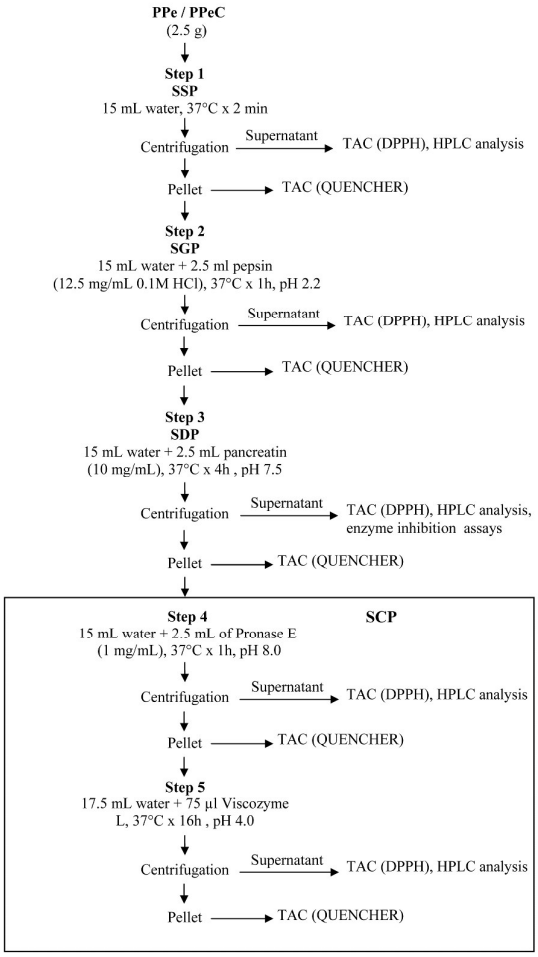

665

666 **Figure 1**

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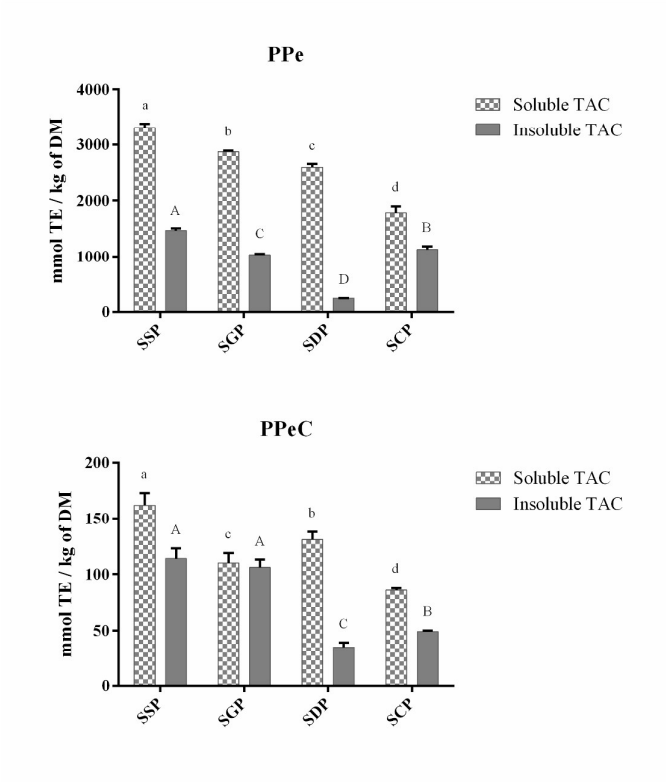

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669 **Figure 2**

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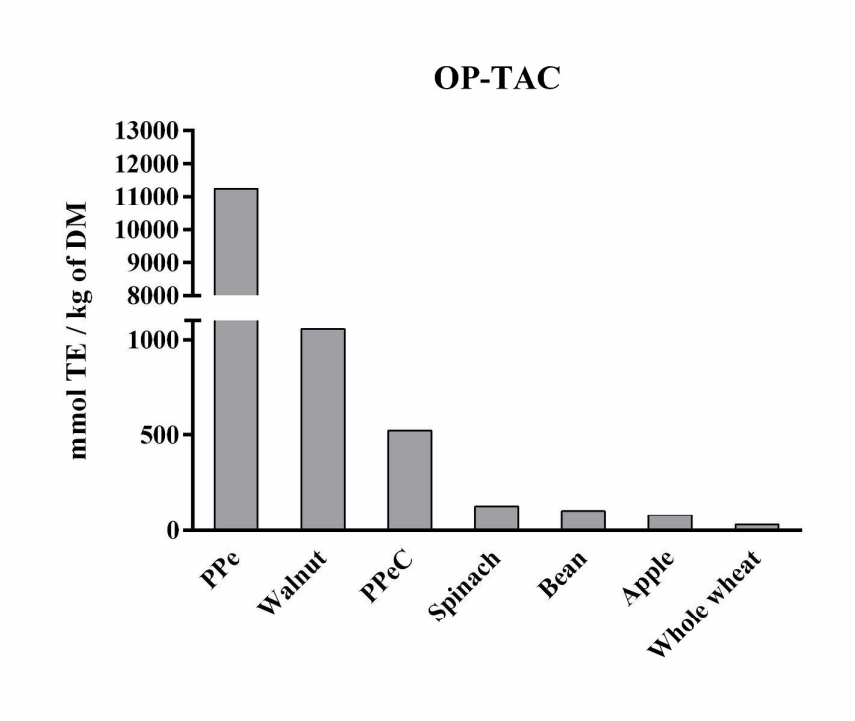

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672 **Figure 3**

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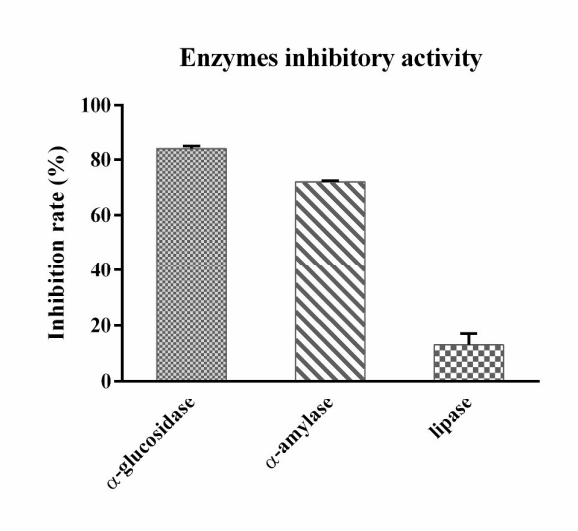

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676 **Figure 4**

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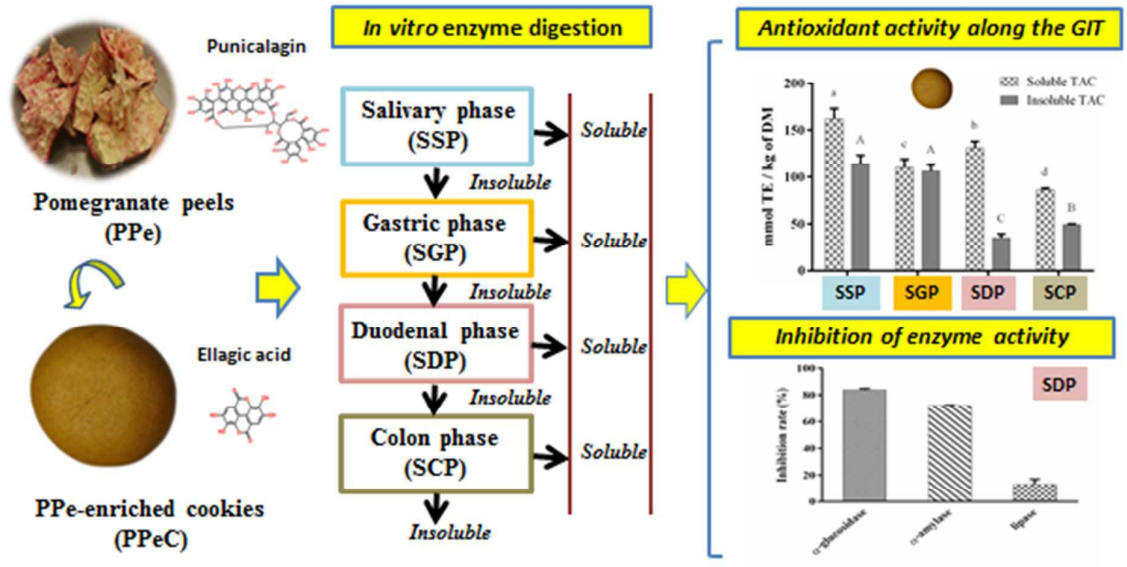
Graphical Abstract

Pomegranate peels (PPe) were proposed as a functional ingredient with a high potential to reduce oxidative processes along the gastro-intestinal tract and to modulate carbohydrate and lipid metabolism.



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