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Hypoglycemic, Antiglycation and Cytoprotective Properties of A Phenol Rich Extract from Waste Peel of *Punica Granatum* L. Var. Dente Di Cavallo DC2

Antonella Di Sotto [1,*], Marcello Locatelli [2,*], Alberto Macone [3], Chiara Toniolo [4], Stefania Cesa [5], Simone Carradori [2], Margherita Eufemi [3], Gabriela Mazzanti [1] and Silvia Di Giacomo [1]

[1] Department of Physiology and Pharmacology, Sapienza University, P.le Aldo Moro 5, 00185 Rome, Italy
[2] Department of Pharmacy, University "G. D'Annunzio" of Chieti-Pescara, Via dei Vestini 31, 66100, Chieti, Italy
[3] Department of Biochemical Sciences, A. Rossi Fanelli, Sapienza University, P.le A. Moro 5, 00185 Rome, Italy
[4] Department of Environmental Biology, Sapienza University, P.le Aldo Moro 5, 00185 Rome, Italy
[5] Department of Chemistry and Technology of Drugs, Sapienza University, P.le Aldo Moro 5, 00185 Rome, Italy
* Correspondence: antonella.disotto@uniroma1.it; marcello.locatelli@unich.it

Abstract: Pomegranate peel is a natural source of phenolics, claimed to possess healing properties, among which antioxidant and antidiabetic. In line with this evidence, the ethyl acetate PGE extract, obtained by Soxhlet from the peel of Dente di cavallo DC2 variety and characterized by a 4% amount of ellagic acid, has been studied for its hypoglycemic, antiglycation and antioxidative cytoprotective properties, in order to support a possible further nutraceutical interest. The α-amylase and α-glucosidase enzyme inhibition, interference with advanced glycation end-products (AGE) formation and metal chelating abilities were evaluated as hypoglycemic mechanisms. Also, considering that oxidative stress is associated with hyperglycemia complications, PGE antioxidant cytoprotective properties under hyperglycemic conditions were assayed. Phenolic profile was characterized by integrated chromatographic and spectrophotometric methods. Under our experimental conditions, PGE strongly inhibited the tested enzymes, especially α-glucosidase, and exerted chelating and antiglycation properties. Also, it reduced both ROS and GSH levels under hyperglycemic conditions, thus suggesting its ability to support cell functions by counteracting intracellular oxidative stress. Along with ellagic acid, rutin was the major identified flavonoid (about 4 %) of PGE. Present results suggest PGE to be a possible remedy for hyperglycemia management and encourage further studies to exploit its promising properties.

Keywords: vegetable waste; phenolics; hyperglycemia-induced oxidative stress; antioxidant activity; AGE inhibition

1. Introduction

Fruit and vegetable waste (i.e. seeds, peel, rind, and pomace) are defined as the inedible parts of vegetables, discarded during collection, handling, transportation and processing [1]. Waste management is an important issue of the modern society, due to its environmental and economic impact. It has been reported that up to 87% of fruits, vegetables and cereals are discarded before reaching consumers [2], thus posing disposal and environmental problems. Nevertheless, waste could be reduced by applying targeted prevention strategies, among which reduction, reuse and recycling [2]. Particularly, recycling strategies (e. g. composting, processing to flour, conversion into water) allow to recovery waste after major modifications and to reuse for further applications or as starting material for extraction of specific compounds [3].

Bioactive phytochemicals, among which polyphenols, anthocyanins, carotenoids, terpenoids, glucosinolates and dietary fibers, represent potentially valuable targets for recycling strategy, with a great interest in drug discovery and nutraceutical fields [4-10]. Polyphenols are a large class of secondary metabolites, with diverse chemical structures and functions, usually concentrated in rind, peel, and seeds of fruits and vegetables [4]. Often fruit peels (e.g. banana, apples, peaches, and pears) have been found enriched in phenolics respect to the edible portions [11].

Among fruit waste, pomegranate peel is known to be an interesting phenolic source with an amount about a ten-fold higher than that of the pulp [12]. All the parts of pomegranate plant, including edible part, juice and nonedible peel, seeds and flowers have been widely assessed for the possible healing effects, thus highlighting antimicrobial, antioxidant, antinflammatory, antiproliferative, hypolipidemic and hypoglicemic properties [13-17]. In line with this evidence, peel byproducts have been approached as possible natural additives for food preservation and quality enhancement, along with as components of food supplements and nutraceuticals in order to exploit their health promoting features [13].

The potential healing effects of pomegranate byproducts are mainly ascribed to the polyphenolic compounds, among which ellagitannins (i.e. punicalagins, punicalin and gallagic acid), phenolic acids (i.e. gallic acid and ellagic acid), anthocyanins and flavonoids, whose amount can differ as a consequence of environmental factors, extraction methods and varieties [13].

In line with this evidence, present study was aimed at evaluating the possible healing properties of an ethyl acetate extract obtained by Soxhlet from the pomegranate (*Punica granatum* L.) peel of the Italian variety Dente di Cavallo DC2 (PGE).

Particularly, we focused on the potential in vitro hypoglycemic and antiglycation properties of PGE, in terms of inhibition of α-amylase and α- glucosidase, key enzymes responsible for dietary carbohydrates digestion, along with the interference with the formation of advanced glycation end-products (AGE) and metal chelating abilities. This study was supported by the previous evidence that PGE was characterized by a higher amount of ellagic acid than punicalagins (5.4: 1 ratio), with low amounts of anthocyanins (about 0.002 % w/w) [18] and that phenolic acids are known to regulate carbohydrate metabolism and hepatic glucose homeostasis through different mechanisms [19-20].

Taking into account that PGE was also reported to possess antioxidant and radical scavenging properties [18], the ability of PGE to exert cytoprotective effects towards the oxidative stress associated with hyperglycemia, has been evaluated. Phenolic profile of PGE was furtherly characterized by integrated chromatographic techniques (HPTLC and HPLC) and spectrophotometric assays, in order to identify the possible bioactive phytochemicals.

2. Results and Discussion

2.1. Phytochemical analysis

Spectrophotometric analysis showed PGE contained high levels of total phenolics and tannins, expressed as μg equivalents of tannic acid (TAE) per milligram of extract (Table 1): these data are in agreement with the previous characterization that highlighted PGE to contain the greatest amount of phenolics respect to the ethanolic and methanolic pomegranate peel extracts [18].

Table 1. Amounts of total phenolics, tannins and flavonoids in the ethyl acetate extract by Soxhlet of the waste peel of *Punica granatum* L. var. Dente di Cavallo DC2 (PGE) (n = 6).

Compound	PGE μg/mg sample (mean ± SE)
Phenolics (TAE)	206.3 ± 0.01
Tannins (TAE)	52.9 ± 0.01
Flavonoids (QE)	205.6 ± 14.70

TAE, chlorogenic acid; QE, quercetin equivalents.

Considering that we found a phenolics/tannins ratio of 3.9 w/w, tannins can be estimated to be about a 40% of total phenolics. Also, our results highlighted that PGE contained about a 20% w/w of total flavonoids (expressed as quercetin equivalents). Taking into account the overall yield obtained for PGE (about 1.09 % w/w) [18], a total flavonoid amount in the fresh material higher than 2 g/Kg can be estimated: on the basis of Peterson and Dwyer classification [5], peel of Dente di Cavallo DC2 pomegranate results to contain high flavonoid levels, thus confirming its possible interest as a nutraceutical source. Nevertheless, the true bioavailability of these phytochemicals after ingestion remain to be evaluated.

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The presence of different phenolics in PGE was also highlighted by HPTLC analysis, that were evidenced as fluorescent spots and visualized by derivatization (Figure S1): among them, gallic acid and catechin were identified (Figure 1).

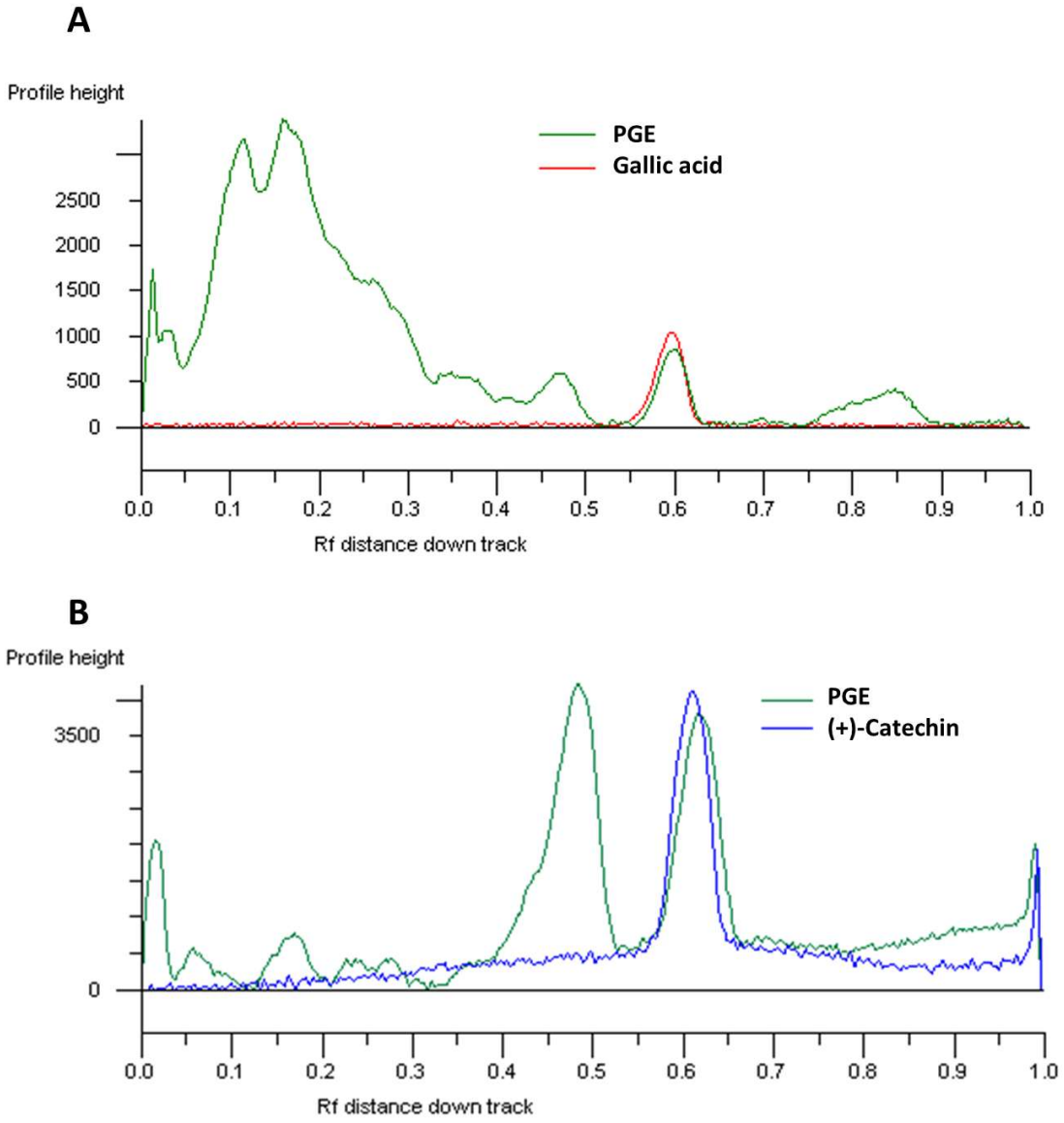

Figure 1. HPTLC chromatogram of the ethyl acetate extract, obtained by Soxhlet from the waste peel of *Punica granatum* L. var. Dente di Cavallo DC2 (PGE), and the standard phenolics gallic acid (A; Rf 0.58 - UV 366 nm after NPR derivatization) and (+)-catechin (B; Rf 0.58 –UV 366 nm after derivatization with NPR and anisaldehyde).

Phenolic profiles were also detected and quantified by HPLC-DAD analysis, whose results agreed with the integrated analysis reported above (Table 2). Particularly, PGE resulted to contain high amount of rutin (about 4% w/w), along with catechin (about 0.27% w/w), 2,3-diMeO benzoic acid (about 0.2% w/w), gallic acid (about 0.1% w/w) and syringic acid (about 0.06% w/w). As previously reported [18], PGE also contained about a 4% w/w of ellagic acid and 0.7% of total punicalagins (i.e. anomers α and β), with low levels of anthocyanins (0.002% w/w). This phytochemical profile highlighted a peculiar 1:1 ratio between the most abundant phenolics, ellagic acid and rutin.

Table 2. HPLC- PDA analysis of the phenolic composition of the ethyl acetate extract, obtained by Soxhlet from the waste peel of *Punica granatum* L. var. Dente di Cavallo DC2 (PGE) (n = 3).

Compound	PGE μg/mg of sample (mean ± SD)
3-OH-4-MeO Benzaldehyde	nr
Benzoic acid	nr
2,3-diMeO Benzoic acid	1.84 ± 0.11
3-OH Benzoic acid	nr
p-OH Benzoic acid	nr
Carvacrol	nr
Catechin	2.65 ± 0.11
Chlorogenic acid	nr
t-Cinnamic acid	nr
o-Coumaric acid	nr
p-Coumaric acid	nr
t-Ferulic acid	nr
Gallic acid	1.08 ± 0.09
Harpagoside	nr
Naringenin	nr
Naringin	nr
Quercetin	nr
Rutin	41.2 ± 3.8
Sinapinic acid	nr
Syringic acid	0.60 ±0.06
Vanillic acid	nr

nr, not revealed.

Published literature highlighted that peel of pomegranate is characterized by a peculiar pool of phenolics (predominantly those from hydrolysable tannins), with punicalagin and its metabolites as the major and most studied compounds. Particularly, the amount of hydrolysable tannins has been reported to be in the range of 27–172 g/kg, with a prevalence of monomeric phenolics [21]. Tannins are mainly characterized by gallic acid and ellagic acid esters (i.e. gallotannins and ellagitannins), including the gallagylesters punicalin and punicalagin, with lower amounts of hydroxybenzoic acids [21]. Li et al. [22], in the peel from Chinese pomegranate, identified catechin, epicatechin and low levels of rutin, chlorogenic and caffeic acid along with punicalagin, ellagic and gallic acid. Accordingly, gallic acid, ellagic acid, caffeic acid, p-coumaric acid, quercetin, and vanillic acid were found to be the predominant compounds in the peel from Tunisian varieties of pomegranate [23]. Phenolic composition of the extracts from pomegranate peel can varied also as a consequence of extraction method. They have been usually extracted using methanol, ethanol or hydroalcoholic solutions [24-25], or water under elevated temperature and extended time, with possible consequent oxidation of certain phenolics [26]. Also, applying specific methodologies, such as sonication, supercritical CO2, increasing extraction temperatures, microwave, ultrasound-assisted extraction can improve the yield of total phenolics [26-30]. Wang et al [26] reported that methanol, ethanol, acetone, and water generally yielded a significant co-extraction of phenolics, proanthocyanidins and flavonoids, while decreased the yield of target antioxidants; nevertheless, ethyl acetate seems to favor the selective extraction of peculiar antioxidants.

In line with this evidence, the extraction by Soxhlet in ethyl acetate of the peel from "Dente di Cavallo DC2" pomegranate allowed to obtain high levels of ellagic acid, similarly to maceration in ethyl acetate, despite a significant lowering of punicalagins (about 0.2:1 ratio between punicalagins and ellagic acid) [18].

Conversely, the ethanolic extract was most abundant in punicalagins, with lower amount of ellagic acid (about a 14:1 ratio between punicalagins and ellagic acid).

Accordingly, partitioning between water and ethyl acetate has been reported to increase ellagic acid content along with the antioxidant potency of the pomegranate peel extracts [31].

This confirms that extraction in ethyl acetate by Soxhlet could represent a suitable method for selection of bioactive phenolic acids and flavonoids from pomegranate peel.

2.2. Metabolic enzyme inhibition

Multiple evidence highlighted that dietary phenolic seems to be responsible for antidiabetic effects, likely due to the ability of these compounds to regulate key pathway of carbohydrate metabolism [32]. Some phenolics has been reported to decrease the activity of α-amylase and α-glucosidase, two important enzymes responsible for digestion of dietary carbohydrates to glucose [33]. Particularly, salivary and pancreatic α-amylases catalyze the endo-hydrolysis of α-1,4-glucosidic linkages of amylose, while α-glucosidases are responsible for further digestion of terminal α-1,4-glucosidic linkages in the small intestinal brush border, with glucose release and absorption across the intestinal enterocytes via specific transporters [33]. Therefore, inhibiting these enzymes can allow to reduce the rate of glucose release in the small intestine and to suppress postprandial hyperglycemia and formation of glycated end-products, with further complications.

In line with this evidence and taking into account the peculiar phenolic composition of PGE, the ability of the extract to affect the function of both enzymes as possible mechanisms for glycemia control, has been studied. Under our experimental conditions, PGE was found able to inhibit the α-amylase enzyme in a concentration-dependent manner, being effective already at lower concentrations, likewise the positive control acarbose (Figure 2). On the basis of the IC50 values, the potency of PGE resulted about 2.5 folds higher than that of acarbose (Table 3).

Also, PGE produced a marked and concentration-dependent α-glucosidase inhibition, achieving the maximum effect at 10 μg/mL (Figure 3). The effect was similar to that produced by the positive control acarbose, as confirmed by the IC50 values (Table 3). Conversely, the extract resulted ineffective towards lipase enzyme, thus suggesting its selective activity for enzymes involved in carbohydrate metabolism (Table 3).

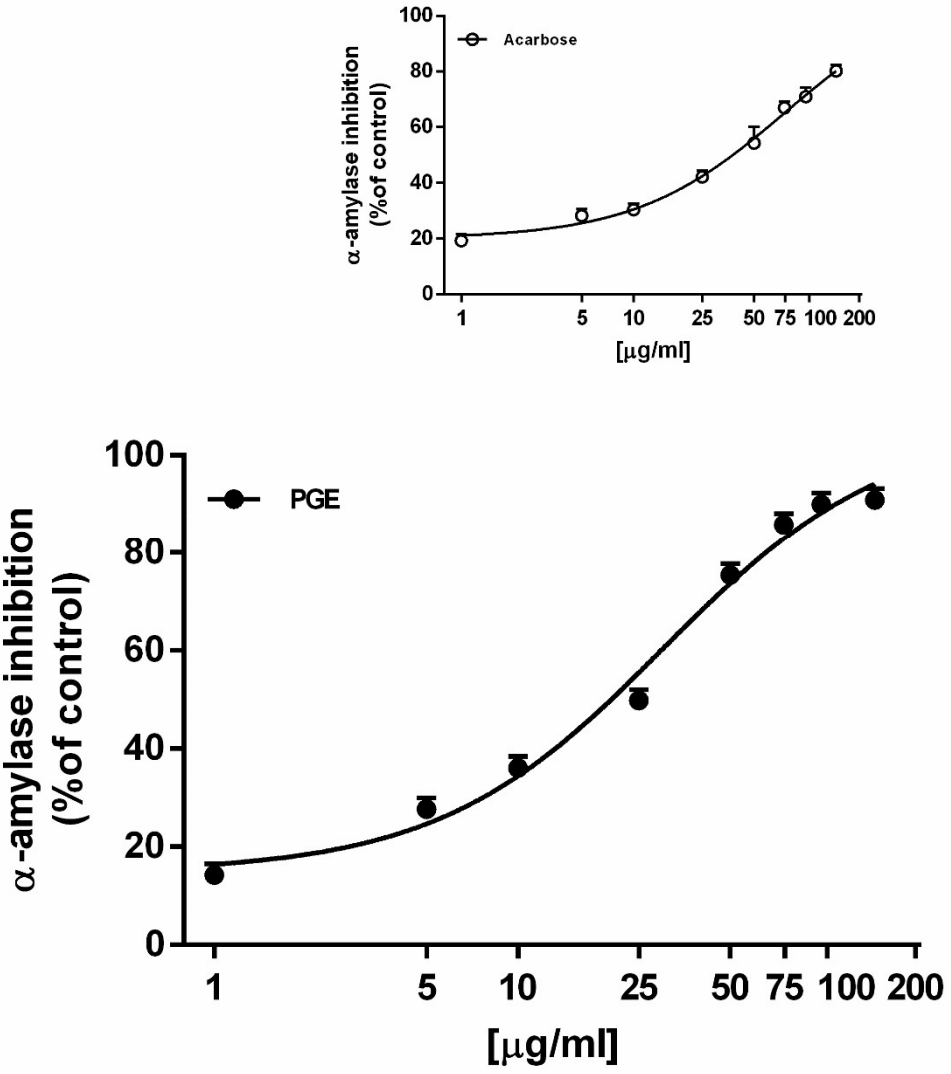

Figure 2. α-Amylase inhibition by the ethyl acetate extract, obtained by Soxhlet from the waste peel of *Punica granatum* L. var. Dente di Cavallo DC2 (PGE), respect to the reference standard acarbose. Each value represents mean ± SEM (n=4).

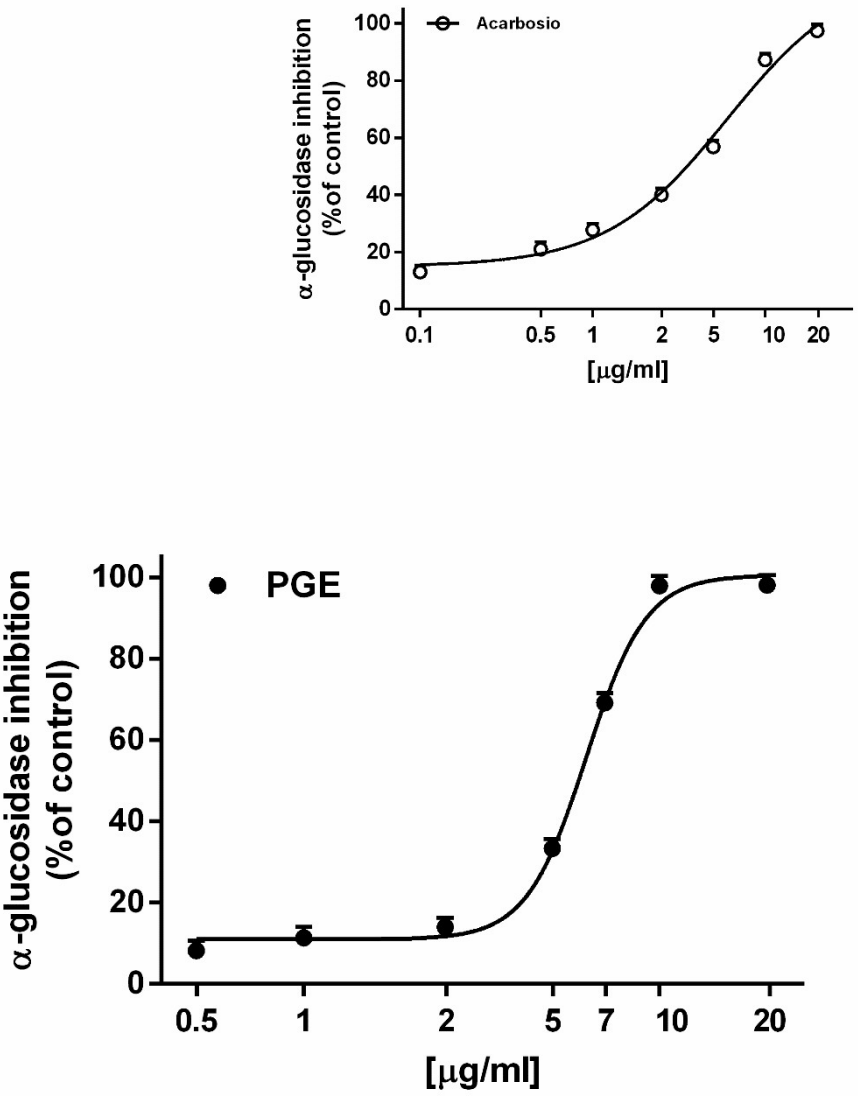

Figure 3. α-Glucosidase inhibition by the ethyl acetate extract, obtained by Soxhlet from the waste peel of *Punica granatum* L. var. Dente di Cavallo DC2 (PGE), respect to the reference standards acarbose and quercetin. Each value represents mean ± SEM (n=4).

Taking into account the highest potency of PGE as α-glucosidase inhibitor and considering that several flavonoids have been reported to inhibit the enzymes involved in the carbohydrate metabolism [34-35], we also assessed the possible contribution of rutin to PGE activity, being the most abundant identified flavonoid. Under our experimental conditions, rutin significantly inhibited α-glucosidase enzyme, being the potency similar to those produced by acarbose and PGE, as confirmed by the IC50 values (Table 3). Our results agree with literature, which highlighted rutin to possess glycolytic enzyme inhibitory and antiglycation potential properties [36-37]. It also exhibited an acarbose-like and specific inhibitory effect on maltase activity in the intestine, and reduced glycemia when administered before glucose overload [38]. Although polyglycosylation is known to decrease the flavonoid ability to inhibit α-amylase and α-glucosidase, rutin has been reported to deactivate the enzymes by forming inactive complex [39] and to be more effective than the derived flavonol quercetin [35]. In regard to the minor components of PGE, the contribution of punicalgins seems to be of lower relevance, taking into account that previous data highlighted the ethanolic extract from the peel of Dente di Cavallo DC2 pomegranate, characterized by a high amount of punicalagins respect to ellagic acid (14:1 ratio)

[18], was ineffective as α-glucosidase inhibitor (data not shown). Altogether, this evidence supports our hypothesis about the possible contribution of rutin to the PGE activity.

Table 3. IC50 values of the ethyl acetate extract, obtained by Soxhlet from the waste peel of *Punica granatum* L. var. Dente di Cavallo DC2 (PGE), and the positive controls for enzyme inhibition, AGE inhibition and chelating activity.

	PGE	Positive controls
	IC_{50} (CL) μg/mL	
α-Amylase inhibition	30.3 (13.4 – 67.3)	76.3 (46.2 – 126.0) [a]
α-Glucosidase inhibition	6.2 (5.8 – 6.7)	6.0 (2.7 - 9.4) [a]
		7.3 (4.2 - 11.8)[b]
Lipase inhibition	nd	6.6 (1.4 – 12.3)[c]
Ferrous ion chelating activity	18.2 (17.4 – 19.1)	360.5 (299.9 – 486.8) [b]
Ferric ion chelating activity	22.3 (7.6 – 69.1)	68.5 (54.9 – 85.7) [d]
AGE inhibition	142.3 (108.5 – 186.7)	41.5 (35.9 – 48.0) [b]
		86.0 (77.3 – 97.5) [e]

nd, not determinable as the inhibition was lower than the 40%. [a] Acarbose; [b] rutin; [c] orlistat; quercetin [d]; [e] naringenin.

On the basis of the Pearson correlation analysis, the inhibition of these enzymes by PGE appeared to be not significantly correlated, being the extract about five-fold more potent in inhibiting α-glucosidase (Table 4). This suggest that PGE is able to affect the activity of both enzymes, although different compounds or inhibitory mechanisms can be responsible for the higher potency as α-glucosidase inhibitor.

Among the other identified compounds of PGE, gallic acid has been reported to affect both α-amylase and α-glucosidase [40] and to be useful to reduce side effects of acarbose when used in combination [41]. Conversely, Kam et al. [42] highlighted that gallic and ellagic acids were effective towards α-glucosidase, while producing only a weak inhibition of α-amylase. *In silico* studies also highlighted catechin and syringic acid to possess inhibitory properties towards α-glucosidase and α-amylase, respectively [34].

Taken together, this evidence suggest that PGE contains a pool of phenolics able to differently affect several targets: particularly, the high potency against glucosidase seems to be ascribable to the combined contributions of rutin, catechin, ellagic and gallic acid, while rutin and syringic acid could contribute to the amylase inhibition. Nevertheless, tangled interactions among compounds in the PGE phytocomplex cannot be excluded.

Furthermore, the high potency of PGE as an α-glucosidase inhibitor is noteworthy and promising, considering that α-glucosidase inhibitor drugs are reported to be useful for adequate control of type 2 diabetes, despite the side effects and costs that limit their market [43]. In this context, PGE could represent a suitable natural alternative to the available treatments for the management and prevention of diabetes and associated ailments to be further studied and developed.

Table 4. Pearson correlation coefficient among biological activities of PGE.

	Pearson r (CL; R square)				
	α-amylase inhibition	**α-glucosidase inhibition**	**Fe^{+2}- reducing activity**	Fe^{+3}- reducing activity	AGE inhibition
α-amylase inhibition	1	-	-	-	-
α-glucosidase inhibition	nsc	1	-	-	-
Fe^{+2}- chelating activity	0.99* (0.4 – 0.99; 0.99)	nsc	1	-	-
Fe^{+3}- chelating activity	0.99* (0.6 – 0.99; 0.99)	nsc	0.99** (0.97– 0.99; 0.99)	1	-
AGE inhibition	nsc	nsc	0.91* (0.15– 0.99; 0.83)	0.93* (0.28 – 0.99; 0.87)	1

* $P < 0.05$ or ** and $P < 0.01$, statistically significant correlation (two-tailed t-test).
nsc, not significantly correlated. CL, Confidential limits.

2.5. Iron chelating activity

Alterations of iron and copper homeostasis are peculiar features of diabetes, evidenced by deposition of iron and copper in different tissues and increased urinary excretion. Therefore, chelating therapies have been suggested as possible strategies to counteract metal-catalyzed oxidation reactions and ROS production, though blocking the Fenton cascade, thus preventing the development of hyperglycemia complications [44]. In line with this evidence, in the present study PGE exhibited similar ferrous and ferric ion chelating activities, achieving about the maximum effects of 80% at the concentration of 100 μg/mL (Figure 4): accordingly, the IC50 values were not statistically different (Table 4).

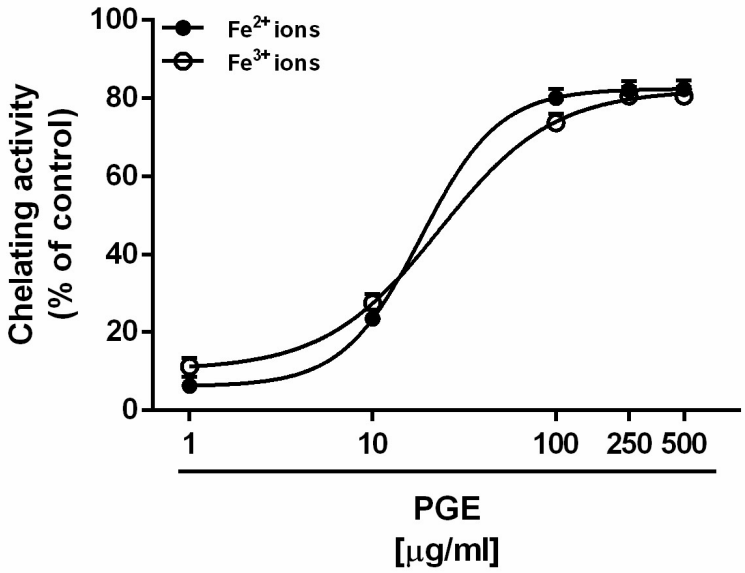

Figure 4. Chelating activity exhibited by the ethyl acetate Soxhlet extract of waste peel of *Punica granatum* L. var. Dente di Cavallo DC2 (PGE) towards ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions. Each value represents mean ± SEM (n=4).

According to the results of Pearson analysis, the chelating abilities of PGE towards ferrous and ferric ions appear to be correlated between them, although were not correlated with the glycolytic enzyme inhibition (Table 4). Also, a significant correlation was found with the inhibition of AGE formation, as expected (Table 4). Interestingly, the extract was about 20- and 3- fold more potent than the standard flavonoids rutin and quercetin, respectively. This suggests that the PGE phytocomplex can contain more potent bioactive compounds or that possible synergistic or additional effects among its constituents allow to improve the activity respect to the pure compounds.

Among the identified compounds, ellagic acid was reported to be able to form complexes with several metal ions and to produce antioxidative effects through reducing the iron-mediated free radical formation by chelating mechanisms [45]. Also, the free radical scavenging effects of rutin and the inhibition of lipid peroxidation were ascribed to its ability to chelate iron ions, thus leading to the formation of metal-complexes that slightly promote free-radical reactions [46-47]. Interestingly, catechin was found to exert cytoprotective and antiradical activities likely through its iron-chelating abilities [48-49]. At last, for gallic and syringic acid a lower iron-binding efficiency, likely due to the chemical features, was reported [50-51].

On the basis of this evidence, different phenolics, among which ellagic acid, rutin and catechin seem to likely contribute to the iron chelating abilities of PGE.

2.6. AGE inhibition

The formation of advanced glycation end-products (AGE) results from a non-enzymatic reaction between the carbonyl group of reducing sugars and a free amino group of proteins, to form reversible Schiff's base, which undergoes rearrangement to Amadori products, which in turn undergo a series of modifications to form stable, heterogeneous adducts [52]. Moreover, a variety of other pathways such as autoxidation of glucose, or ascorbate and lipid peroxidation, can lead to AGE formation [53]. AGE formation deserved special attention due to its involvement in development and progression of different pathologies (such as atherosclerosis, nephropathies and retinopathy), particularly diabetes complications [52, 54]. Particularly, they are considered to be glycotoxins, due to their ability to increase oxidant stress and inflammation [55]. Among the proposed mechanisms for AGE damage, they seem to activate specific cellular receptors, namely RAGE, which have been found highly expressed during pathogenic and stress conditions, thus inducing the gene expression of cell specific pro-inflammatory and oxidative signalling pathways [56]. Also, under oxidative stress, tissue accumulation of AGE has been reported to cause up-regulation of the matrix metalloproteinases, which are associated with chronic inflammatory conditions [55]. Therefore, inhibiting AGE formation, at different steps of glycation process [55], has been highlighted to represent a possible therapeutic target for the prevention and treatment of different chronic pathologies, particularly diabetes complications.

Under our experimental conditions, PGE produced a marked and concentration-dependent inhibition of AGE, achieving the maximum effect at 1000 μg/mL (Figure 5). However, its potency was about 1.7 folds lower than the positive control naringenin, as confirmed by the IC50 value (Table 3).

Rutin, the most abundant flavonoid identified in PGE, produced a concentration-dependent inhibition of the AGE levels (Figure 6), with about a three-folds higher potency than PGE (Table 3): it was also effective as AGE inhibitor at concentration found in PGE, thus suggesting a possible contribution to the activity of the extract.

The Person correlation analysis highlighted that AGE inhibition by PGE was not significantly correlated with inhibition of glycolytic enzyme, despite a significant correlation with chelating activity (Table 4). Similarly, the AGE inhibition by rutin was not significantly correlated with its glycolytic enzyme inhibition. This behaviour supports our hypothesis about the contribution of rutin to the PGE bioactivity, although the involvement of all the phytocomplex appears to be likely.

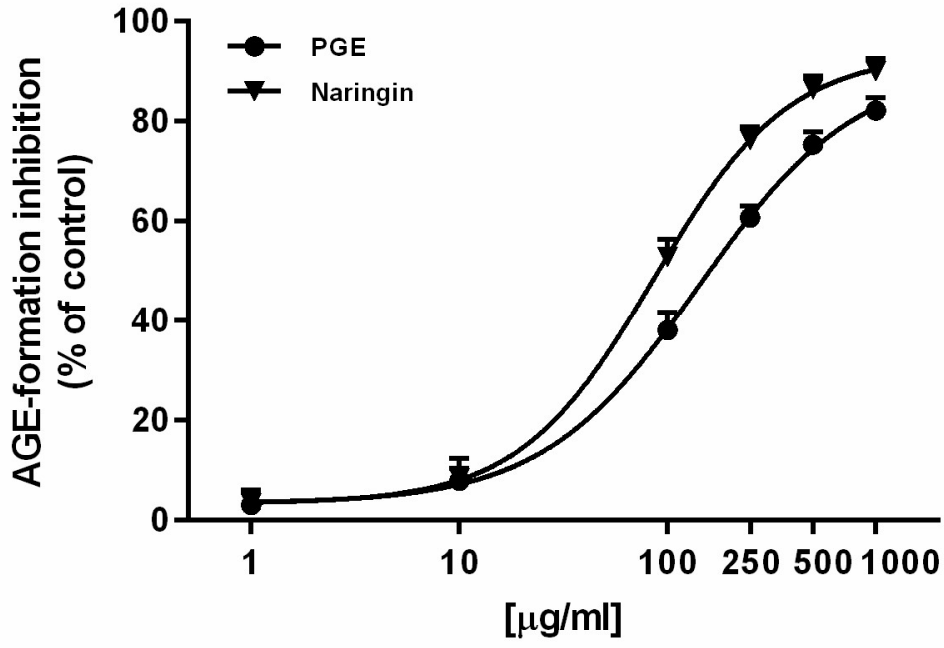

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Figure 5. AGE inhibition by the ethyl acetate extract, obtained by Soxhlet from the waste peel of *Punica granatum* L. var. Dente di Cavallo DC2 (PGE), and the positive control naringenin. Each value represents mean ± SEM (n=4).

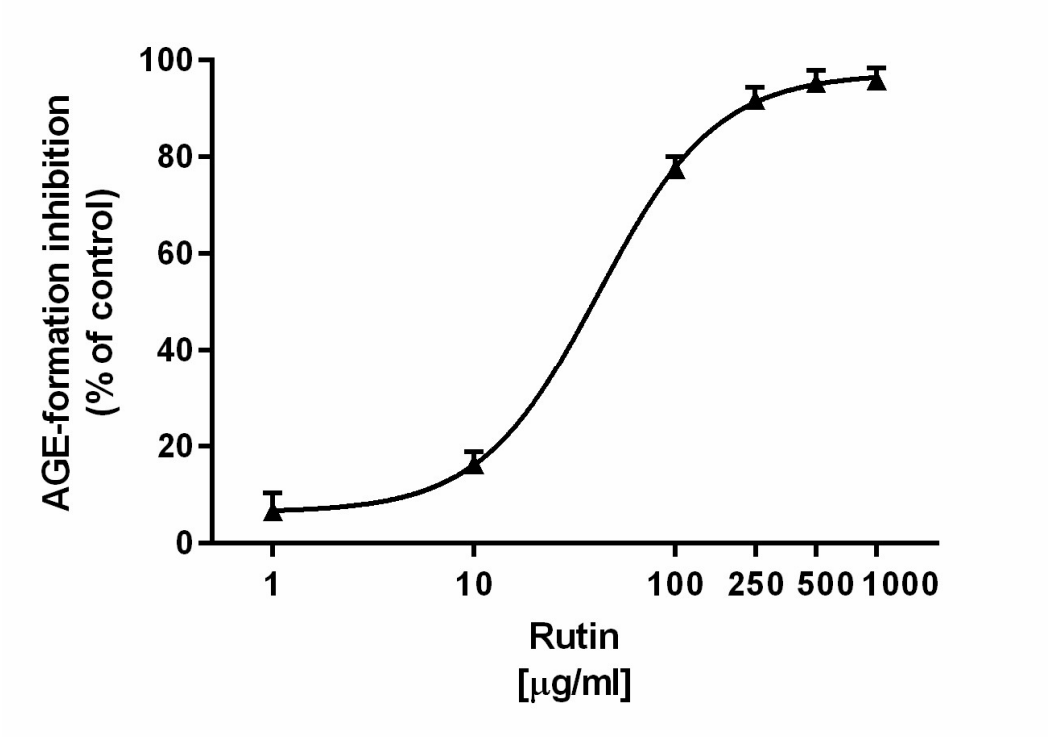

Figure 6. AGE inhibition induced by rutin. Each value represents mean ± SEM (n=4).

Our results agree with previous published literature, which highlighted rutin and its derived flavonol to possess antiglycation *in vivo* properties, being able to prevent AGE formation, through free-radical scavenging and metal chelating mechanisms [57]. Particularly, structure-activity relationship (SAR) studies highlighted that the presence of vicinyl dihydroxyl groups in the B-ring of flavonoid is required for the anti-glycation effects of rutin through free-radical scavenging activity [55]. Moreover, gallic acid and catechin have been reported to prevent the AGE formation by trapping α-dicarbonyl compounds or inhibiting Amadori product formation [58-59]. The antiglycation activity of some polyphenols, among which gallic acid, seems to be mediated by the upregulation of PPAR (peroxisome proliferator-activated receptors) nuclear receptors, which play a pivotal role in the control of carbohydrate and lipid metabolisms and in the downregulation of RAGE expression [53]. Particularly, a pomegranate flower extract rich in gallic acid showed to increase the PPAR-gamma mRNA expression along with enhancing the sensitivity to insulin receptor, thus suggesting that this mechanism could be involved in the control of AGE formation [60]. Similarly, ellagic acid exhibited interesting antiglycation properties both in vitro and in vivo, by reducing the expression of RAGE along with that of specific regulatory factors for angiogenesis and hypoxia, thus suggesting it can represent a potent antiglycating agent to be further developed [61]. This evidence, in agreement with the previous described antioxidant, glycolytic enzyme inhibitory and chelating properties, strengthen our hypothesis about a possible role of PGE phytocomplex in the control of hyperglycemia-related ailments and suggest the need of further in vivo evaluation of its potential usefulness in diabetic animal models.

2.7. PGE counteracts the oxidative stress induced under hyperglycemia conditions

Recent evidence has suggested that diabetes can be considered as an oxidative stress disorder, occurring as a consequence of an imbalance between free radical formation and reduced ability of the

physiological antioxidant defences [62]. Oxidative stress also occurs as a consequence of mitochondrial dysfunction and alternative metabolism of glucose (mainly polyol pathway) under hyperglycemic conditions, and in turn, contributes to diabetes complications [63]. Therefore, free radicals can oxidize cell structures, including protein, lipid, and nucleic acid, and lead to AGE and ALE (advanced lipoxidation end products) formation. AGE accumulation can further contribute to increase oxidative stress and inflammation in the tissues, through the activation of the AGE-RAGE axis and specific pro-oxidant and pro-inflammatory pathways [53,55]. In this context, counteracting oxidative stress during hyperglycemia conditions and diabetes appears to be a suitable strategy to limit the oxidative cascade damage and complication development.

In line with this evidence and taking into account the previous described activities of PGE, we also studied the antioxidant cytoprotective ability of the extract under hyperglycaemic conditions in a gastric cancer cell model, known to be characterized by a glycolytic metabolism [64] and increased oxidative stress under hyperglycaemic conditions [65].

Preliminary cytotoxicity assays showed that PGE did not significantly affect the AGS cells viability up to the concentration of 100 μg/mL and 72 h exposure (data not shown). On the basis of this evidence, this concentration was chosen as the highest to be assessed in the further assays under hyperglycemic conditions. Also, the glucose concentrations of 3.0, 4.5, 6.0 and 7.5 mg/mL were found to progressively increase the intracellular ROS levels respect to basal oxidation levels obtained at a final glucose concentration of 1.4 mg/mL, without affecting cell viability. Particularly, after 72 h incubation, ROS levels increased up from 3.7 to 5.3 folds within the range of the tested concentrations (data not shown). The concentration of 6.0 mg/mL at with a submaximal 5-fold ROS-increase respect to the basal level, was chosen for further cytoprotective assays in the presence of nontoxic concentrations of PGE (i.e. 10. 50 and 100 μg/mL). These data agree with previous published studies, in which 7.8 mM glucose (corresponding to about 1.4 mg/mL) [66] was used as a non-fasting basal blood sugar level, while concentrations of 17.5, 25 and 33 mM glucose (corresponding to about 3.0, 4.5 and 6.0 mg/mL) to simulate hyperglycemic conditions [66-68]. Along with the extract, the most abundant identified phenolics, i.e. ellagic acid and rutin, were assayed at the concentration of 4 μg/mL, corresponding to the level detected in PGE at the highest nontoxic concentration.

Under our experimental conditions, PGE produced a statistically significant and concentration dependent reduction of the ROS levels induced by hyperglycemia (glucose 6 mg/mL), achieving a maximum lowering of about 60% at the highest tested concentration. Likewise, both rutin and ellagic acid produced a similar 63 and 67% lowering of hyperglycemia-induced ROS levels (Figure 7).

Under the same experimental conditions, also total GSH levels were found significantly raised by hyperglycemia, achieving about a 33% increase respect to the basal levels. The treatment with PGE significantly reduced the total GSH levels, achieving a reduction from about 50% to a maximum 80% within the tested concentrations (Figure 8). Similarly, rutin produced a 41% lowering of GSH, despite a lacking effect of ellagic acid at the concentration found in PGE (Figure 8).

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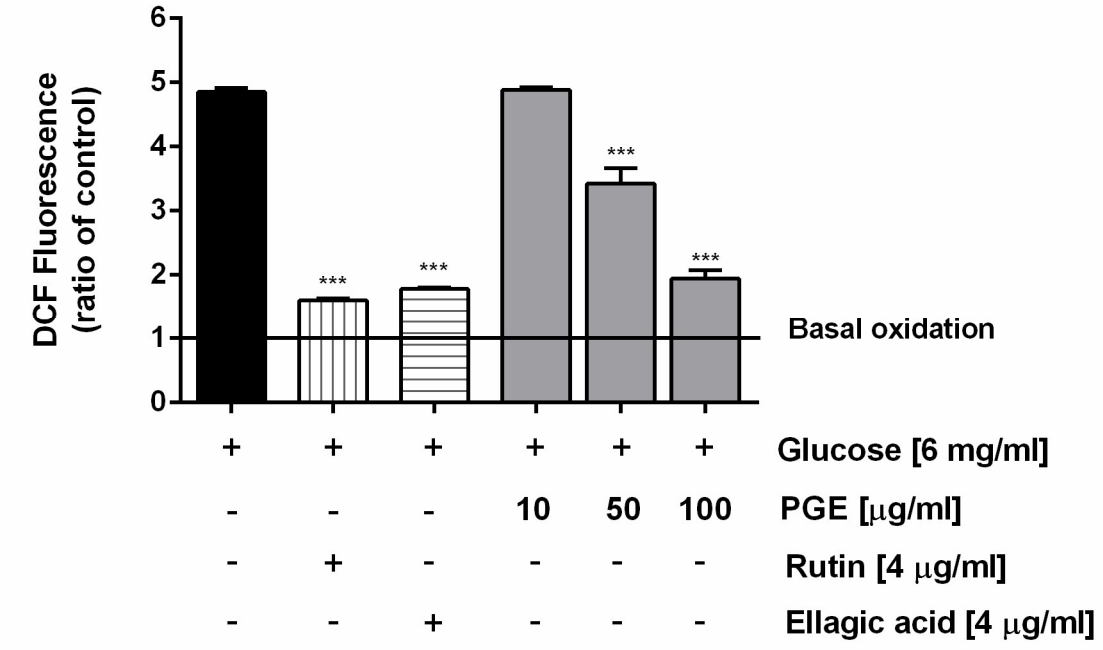

Figure 7. Effect of the ethyl acetate extract, obtained by Soxhlet from the waste peel of *Punica granatum* L. var. Dente di Cavallo DC2 (PGE), rutin and ellagic acid on the ROS levels induced by hyperglycemia conditions in AGS cells. ROS levels are expressed as oxidation index respect to the basal levels. Data are mean ± SE from almost two independent biological replicates (n = 2). *** $p < 0.001$ vs Glucose [6 mg/mL] by ANOVA followed by Dunnett's Multiple Comparison Post Test.

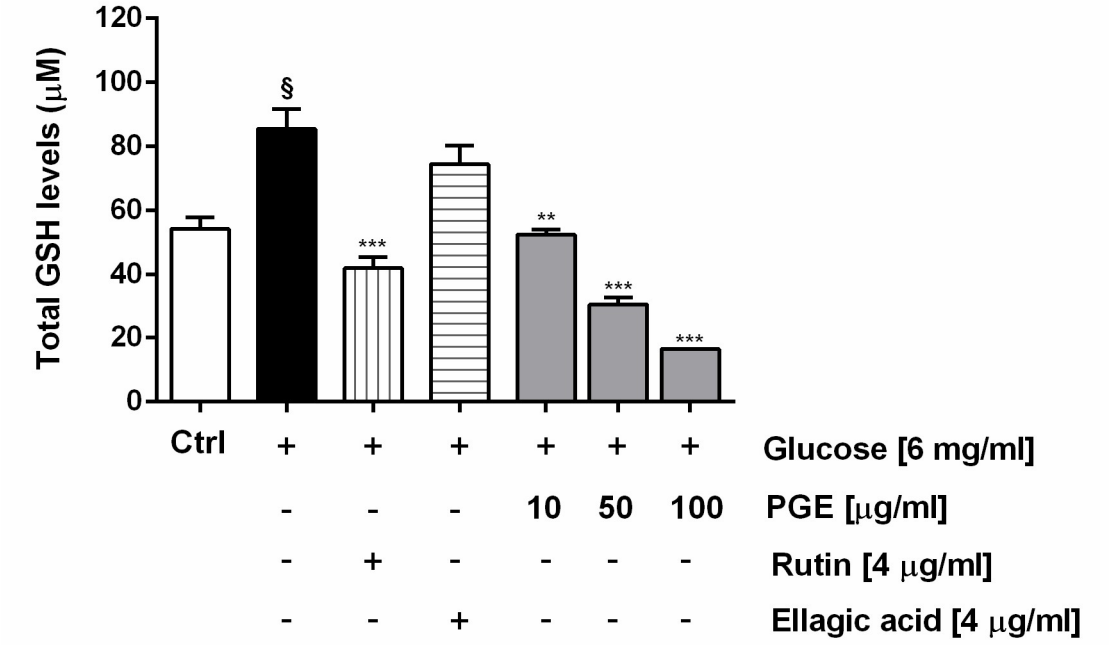

Figure 8. Effect of PGE, rutin and ellagic acid on GSH levels induced by hyperglycemia conditions in AGS cells. GSH was evaluated in cell lysates and calculated respect to the calibration curves of GSH and GSSG. Data are mean ± SE from almost two independent biological replicates (n = 2). § $p < 0.01$ vs control by t-Student test; ** $p < 0.01$ and *** $p < 0.001$ vs Glucose [6 mg/mL] by ANOVA followed by Dunnett's Multiple Comparison Post Test.

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ROS are physiologically produced under aerobic metabolism and their production may be increased during cell injury, like hyperglycemia. Considering that excessive ROS levels may lead cell damage and death, in order to prevent irreversible damage and to restore the redox homeostasis, adaptive cell response, by upregulation of antioxidant systems, occur. Among them, GSH plays a pivotal role in maintaining redox homeostasis and elevated levels have been found in various tumors, likely as a mechanism of cancer cytoprotection and resistance [69]. It is known that antioxidants may directly inhibit ROS by scavenging and inactivating mechanisms, or indirectly by inhibiting their formation, trough metal chelation, or by affecting physiological defences, including reduced glutathione, superoxide dismutase, catalase, and glutathione reductase [70].

Accordingly, under our experimental conditions, GSH and ROS levels were strongly increased by the hyperglycemic oxidative stress in AGS cells, while PGE is able to deplete GSH, likely as a consequence of the reduced ROS levels. Previous evidence highlighted that PGE can act by both radical scavenging mechanisms [18], and through metal chelating properties. Also, under hyperglycemia it resulted able to affect further mechanisms of ROS generation, strictly connected with carbohydrate metabolism and diabetes, by inhibiting glycolytic enzymes and AGE formation. Altogether, this evidence supports our hypothesis about the ability of PGE to counteract the oxidative stress occurring during hyperglycemia, likely acting through direct and indirect antioxidant mechanisms, thus lowering the need of antioxidant cell defences.

Interestingly, both phenolics rutin and ellagic acid were able to affect the ROS levels induced by hyperglycemia in AGS cells, while only rutin was effective in depleting GSH levels at the concentration found in the extract. This behaviour strengthen our hypothesis about the involvement of different phenolics in the PGE bioactivities, and suggest that using the PGE phytocomplex, instead of the pure phenolics, may be a suitable strategy to exploit numerous healing properties along with possible synergistic interactions occurring among the phytochemicals.

3. Materials and methods

3.1. Chemicals

All the chemicals, if not otherwise specified, and the RPMI 1640 medium were obtained from Sigma-Aldrich Co (St. Louis, MO, USA). Sodium carbonate, Folin-Ciocalteu's phenol reagent, tannic acid, aluminium chloride hexahydrate and the analytical grade solvents ethyl acetate (AcOEt) and n-butanol were purchased from Merck (Darmstadt, Germany). Standard flavonoids and phenylpropanoids (>95% purity) were obtained from synthesis and checked by nuclear magnetic resonance spectroscopy.

Fetal bovine serum was obtained from Gibco, while the other reagents for antiviral studies, if not otherwise specified, were purchased from Invitrogen (Carlsbad, CA, USA).

Anisaldehyde was prepared as a 0.5% v/v solution in an ice-cooled mixture of sulphuric acid, methanol and acetic acid (1:17:2 v/v/v). Natural Product Reagent (NPR) was obtained by preparing a 0.5% w/v solution of diphenylborinic acid aminoethylester in AcOEt.

3.2. Plant material and extract preparation

Fruits of Italian Punica granatum L. Dente di Cavallo DC2 cultivar were kindly provided at eating ripeness by the local Italian farm Giovomel (Avellino, Italy).

To prepare the PGE extract, peel (exocarp and mesocarp) of pomegranate fruit was separated from arils, then washed, triturated in a blender for 30 seconds and subjected to Soxhlet extraction in ethyl acetate, according to Masci et al. [18]. The obtained extract was dried at 40 °C in the dark and stored under ultrafrost conditions (-18°C). The extract was dissolved in EtOH for phytochemical and biological analysis.

3.3. Phytochemical analysis

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3.3.1. Determination of total polyphenol and flavonoid content

The total amounts of both phenolics and tannins were measured by the spectrophotometric Folin-Ciocalteau method and calculated as tannic acid equivalents (TAEs), while those of flavonoids were determined by the aluminium trichloride method and expressed as quercetin equivalents (QE), according to previous published methods [5].

3.3.2. Chromatographic analysis of phenolics

Phenolic compounds of PGE were analyses by integrated high-performance thin-layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) according to the methods reported by Di Sotto et al. [5].

Particularly, for the HPTLC, a methanolic solution of PGE (50 mg/mL) was analyzed in comparison with standard phenolics (1 mg/mL), among which phenolic acids (i.e. chlorogenic acid, caffeic acid and gallic acid) flavonoids (i.e. rutin, quercetin and kaempferol), and catechin. Compounds were identified by comparison with selected standards (Rf values, colors, UV spectra) and literature data. Repeatability was determined by running a minimum of three analyses.

For measuring the HPLC-PDA phenolic pattern, PGE was dissolved in the mobile phase at concentration of 1 mg/mL, then directly injected (20 µL) into HPLC-PDA system (HPLC Waters liquid chromatography - model 600 solvent pump, 2996 PDA). According to previous standardized method [5], rutin, naringin, quercetin, harpagoside, naringenin, carvacrol, gallic acid, chlorogenic acid, syringic acid, benzoic acid, p-OH benzoic acid, 2,3-diMeO benzoic acid, 3-OH benzoic acid, 3-OH-4-MeO benzaldehyde, t-cinnamic acid, o-coumaric acid, p-coumaric acid, vanillic acid, sinapinic acid, t-ferulic acid, catechin and epicatechin, were used as standard phenolics.

3.4. In vitro metabolic enzyme inhibition

The ability of PGE to inhibit in vitro the α-amylase, lipase and α-glucosidase enzymes was measured by spectrophotometric assays by using a microplate reader (Epoch Microplate Spectrophotometer, BioTeK). Acarbose (250 µg/mL) and orlistat (25 µg/mL) were included in all the experiments as standard inhibitors (100 % enzyme inhibition) for α-amylase, α-glucosidase and lipase, respectively, while the vehicle represented the maximum enzyme activity. Additional treatments, in which enzyme solution was replaced by buffer solution, were included in order to evaluate the interfering absorbance produced by the extract. The experiments were performed at least in triplicate and in each experiment about six replicates were prepared. Data obtained from at least two experiments were pooled in the statistical analysis. The inhibitory activity was calculated as percentage of inhibition with respect to the vehicle control.

3.4.1. α-Amylase inhibition

The α-amylase activity was determined by the method of dinitrosalicilic acid (DNSA), according to Di Sotto et al. [71], with minor changes. To perform the assay, serial dilutions of the extract (250 µl; 1-100 µg/mL final concentration, 1:2 to 1:5 dilution factor) were pre-incubated with α-amylase (250 µL; 0.5 mg/mL in phosphate buffer solution, corresponding to 25 U/mL) for 10 minutes at 37 °C. Then, the mixtures were added with potato starch solution (0.5 % w/v in acetate buffer 0.1 M, pH 4.5; 250 µL) and incubated for 10 minutes at 37 °C. The reaction was stopped by adding 500 µl of DNSA reagent (25 mL of 96 mM DNSA solution in water, 8 mL of 5.3 M sodium potassium tartrate solution in 2 M sodium hydroxide and 12 mL of water), which is able to bind reducing sugar, thus inducing the turning of the solution to red, after incubation in a water bath at 100 °C for 5 minutes. Then, the DNSA absorbance was measured at 540 nm.

3.4.2. α-Glucosidase inhibition

The α-glucosidase activity was measured through the enzymatic hydrolysis of p-nitrophenyl-α-D-glucopiranoside to p-nitrophenol and D-glucose, according to previous standardized method [71]. To perform the assay, serial dilutions of PGE (25 µL; 0.5 - 10 µg/mL final concentration, 1:1.1 to 1:2.5 dilution factor) were pre-incubated for 30 minutes at 37°C with 50 µL of α-glucosidase (1 U/mL in PBS 0.1 M), and 100 µL of PBS. Then, 25 µL of p-nitrophenyl-α-D-glucopiranoside (5 mM in PBS) were added to each tube and the solution was vortexed for 5 minutes. For each treatment, the p-nitrophenol presence was determined by the turning of the solution to orange and its absorbance was determined at 405 nm.

3.4.3. Lipase inhibition

Lipase activity was measured through the hydrolysis of p-nitrophenyl palmitate to p-nitrophenol, according to Vitalone et al. [72]. Serial dilutions of the samples (16 µL) were mixed with a lipase solution (5.0 mg/mL in water; 12 µL) and TRIS HCl (75 mM, pH 8.5; 162 µL). The mixture was supplemented with PNP (10 mM; 25 µL) and pre-incubated on ice for 5 min. For each treatment, the p-nitrophenol absorbance was determined at 405 nm.

3.5. Iron chelating activity

The activities were evaluated by the ferrozine assay, according to previous published methods [26]. Experiments were performed in acetate buffer (0.1 M, pH = 4.5) to optimize the conditions for ferric ions reduction and chelation [73]. Chelation ability was evaluated against both ferrous and ferric ions. In the first case, a freshly prepared solution of $FeSO_4 \times 7H_2O$ (50 µL; 200 µM) in acetate buffer, was mixed for 2 minutes with the samples (50 µL) and afterwards with acetate buffer (50 µL). Conversely, ferric ions chelating was assessed by mixing a solution of $FeCl_3 \times 6H_2O$ (50 µL; 200 µM) in acetate buffer for 2 minutes with the samples (50 µL), and afterwards with hydroxylamine (50 µL; 5 mM). After addition of ferrozine solution (50 µL; 5 mM), the absorbance of the complex formed with ferrous ions was measured at 562 nm. The percentage of chelating activity was calculated as follows: $100 \times (A_{control} - A_{sample})/A_{control}$, where $A_{control}$ is the absorbance of the vehicle while A_{sample} is that of the tested sample. For each sample, the iron-chelating power was calculated in relation with the positive controls rutin and quercetin.

3.6. Advanced glycation end-product (AGE) inhibition

The inhibition of AGE formation by PGE and the standard phenolics naringenin, rutin and ellagic acid was measured through the method described by Lee et al. [74], with slight modifications. Progressive dilutions of the samples (1–1000 µg/mL of final concentrations for PGE and 0.04 – 20 µg/mL for the pure compounds) were prepared in DMSO, then added to an assay mixture containing bovine serum albumin (150 mM), phosphate buffer (50 mM; pH 7.4), sodium azide (0.02 % w/v), fructose (0.4 M) and glucose (0.4 M). The mixtures were incubated at 37 °C for 7 days, thereafter fluorescence was measured at an excitation wavelength of 355 nm and emission of 460 nm. The inhibitory activity was calculated as percentage of the control, by using the following formula: $(Ac \times As/Ac) \times 100$, where Ac is the fluorescence of the control, and As is the fluorescence of the sample.

3.7. Cytoprotective activity against oxidative stress

3.7.1. Cell culture

The human gastric carcinoma (AGS) cells, kindly given by Prof. Gabriella Mincione (Department of Medical, Oral, and Biotechnological Sciences, University "G. d'Annunzio" of Chieti-Pescara, Italy), were grown at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium, supplemented with fetal bovine serum (10% *v*/*v*), glutamine (2 mM), streptomycin (100 µg/mL), and penicillin (100 U/mL). All experiments were performed when cells reached the logarithmic growth phase.

3.7.2. Cytotoxicity assay

The cells were seeded into 96-well microplates (20,000 cells/well) and allowed to grow for 24 h; then progressive dilutions of the PGE in ethanol (EtOH; 100% *v*/*v*) were added to cells (1% *v*/*v* in the cell medium). The vehicle was nontoxic at final concentration of 1% *v*/v in the medium. Cytotoxicity was measured up to 72 h incubation with the samples by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay according to previous published methods [75]. The assay was carried out three times and, in each experiment, each concentration was tested almost in six technical replicates. Cell viability was determined as follows: [(OD treated cells-OD medium control)/(OD untreated cells-OD medium control)] × 100.

3.7.3. Cytoprotection assay towards oxidative damages

For assessing the cytoprotective activity of PGE towards oxidative damages under hyperglycemia conditions, 5 x 10^5 cells were plated into 6-well culture wells, allowed to grow for 24 h, then confluent cells (about 60-70% of confluence) were further exposed to hyperglycemic conditions (glucose concentration of 6.0 mg/mL) and progressive concentrations of PGE (50, 100 and 500 µg/mL) for 72 h. At the end of incubation, the intracellular levels of reactive oxygen species (ROS) and glutathione, as oxidative stress parameters, were measured as follow.

3.7.4. Determination of intracellular levels of reactive oxygen species (ROS)

The ROS levels induced by treatments were measured by the 2,7-dichlorofluorescein diacetate assay (DCFH-DA), according to Di Giacomo et al. [76] with slight changes. The DCF fluorescence, proportionally induced by ROS-mediated oxidative stress, was measured at an excitation wavelength of 485 nm and emission wavelength of 528 nm by a BD Accuri™ C6 flow cytometer. In each experiment, a vehicle control (corresponding to the basal ROS level) and a positive control, corresponding to the highest oxidation under hyperglycemic conditions, were included too. The mean DCF fluorescence of 10,000 cells was measured from all the treatments. The oxidation index was obtained by the ration between the DCF fluorescence of the sample and that of the vehicle control.

3.7.5. Chromatografic determination of intracellular glutathione levels

Intracellular levels of reduced (GSH) and oxidized (GSSG) glutathione were measured by HPLC-UV, according to previous standardized methods [77]. Briefly, cell pellets (1 × 10^6) were suspended in 10% ice-cold TCA and centrifuged for 15 min at 9000× g. The supernatant was collected and GSH and GSSG were measured by HPLC with UV detection at 215 nm. The separation was achieved using a InfinityLab poroshell 120 EC-C18 column (3 × 150 mm, 2.7 µm) at a flow rate of 0.8 mL/min with the following elution gradient: 0–3 min 100% A + 0% B, 3–10 min from 100% A to 100% B. The composition of mobile phase A was 0.1% trifluoroacetic acid in water and mobile phase B was 0.1% trifluoroacetic acid in water/acetonitrile (93:7). In these chromatographic conditions, retention times were 2.58 min and 7.01 min for GSH and GSSG, respectively.

3.8. Statistical analysis

All values are expressed as mean ± SE. Statistical analysis was performed by GraphPad Prism™ (Version 4.00) software (GraphPad Software, Inc., San Diego, California,USA). The one-way analysis of variance (one-way ANOVA), followed by Dunnett's Multiple Comparison Post Test, was used to analyze the difference between treatments. Moreover, unpaired data were analyzed with Student's t test. The concentration–response curves were constructed using the "Hill equation": E = Emax/ [1 + ($10^{LogEC50}$/A)^{HillSlope}], where E is the effect at a given concentration of agonist, Emax is the maximum activity, IC_{50} is the concentration that produces a 50% of the inhibitory response, A is the agonist

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concentration in molar, HillSlope is the slope of the agonist curve. *P* values of less than 0.05 ($p < 0.05$) were considered statistically significant.

4. Conclusions

The healing effects associated with pomegranate consumption, ascribed to the pleiotropic effects of its secondary metabolites, mainly phenolics, have been widely reported. Also, nonedible pomegranate peel has been highlighted to be enriched in phenolic acid and flavonoids respect to edible parts, thus suggesting it could represent an environmental-friendly nutraceutical source.

Here we reported the hypoglycemic, antiglycation and antioxidant cytoprotective properties of PGE, an ethyl acetate extract obtained by Soxhlet from the waste peel of the Dente di Cavallo DC2 Italian pomegranate variety. The extract was found to be characterized by a peculiar 1:1 ratio between ellagic acid and rutin, which were the most abundant phenolics (about 0.4% w/w), along with lower amounts of catechin, gallic acid, 2,3-dimethoxy benzoic acid, syringic acid and punicalagins.

This composition seems to be correlated with the ability of PGE to inhibit glycolytic enzymes and to affect AGE formation, likely through chelating mechanisms. Indeed, metal chelation has been highlighted to represent a primary mechanism by which inhibitors and breakers of AGE can stop their metal-catalyzed formation. In this context, the PGE chelating activity can contribute to block AGE formation and to counteract the oxidative stress often associated to hyperglycemia. Accordingly, literature data support the possible role of rutin, catechin, gallic and ellagic acids as both AGE inhibitors and metal chelating agents. Moreover, these compounds were reported to inhibit α-amylase and α-glucosidase with different potency, likely due to their specific molecular structures.

Among the identified phenolics, ellagic acid and rutin were also found involved in the antioxidant cytoprotective effects of PGE in AGS cells, being ellagic acid effective in reducing ROS levels, while rutin affected both ROS and GSH amounts. However, considering that synergistic and/or antagonistic interactions can occur under a phytocomplex, the true pharmacodynamic and/or pharmacokinetic contribution of all the PGE phytochemicals remain to be clarified.

It is noteworthy that, also enhancing GSH capacity is an important aim to protect normal cells from redox-related changes or environmental toxins, depleting GSH levels may increase susceptibility of cancer cells to oxidative stress, thus representing a possible strategy for sensitizing cancer cells to chemotherapy. In line with this evidence, further studies about the possible chemosensitizing role PGE in gastric or other cancer cells are encouraged.

Altogether these results provide preliminary scientific evidence about the ability of PGE to counteract oxidative and glycative stress associated with hyperglycemia by pleiotropic mechanisms, thus suggesting further studies to better characterized its possible usefulness as an antihyperglycemic remedy. Also, our results strengthen the interest for the pomegranate peel as a natural source of bioactive phytochemicals or phytocomplexes to be developed as antidiabetic remedies.

Author Contributions:: ADS, ML and SDG conceived and designed the experiments; SC (Stefania Cesa) prepared the Soxhlet extract in ethyl acetate; ADS, ML, SC (Simone Carradori), SDG, SC (Stefania Cesa) and CT performed the phytochemical analysis of the extract; ADS and SDG performed the spectrophotometer analysis, cytotoxicity and cytoprotective studies and inhibition enzyme assays; ME, SDG and AM performed the ROS and GSH analysis; ADS, SDG, AM, ML and CT collected and analyzed the data; ADS, SDG and GM wrote the paper. All authors have approved and revised the final version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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