

RESEARCH ARTICLE

Antiglycative and neuroprotective activity of colon-derived polyphenol catabolites

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Scope: Dietary flavonoids and allied phenolic compounds are thought to be beneficial in the control of diabetes and its complications, because of their ability to inhibit oxidative stress, protein glycation and to act as neuroprotectants. Following ingestion by humans, polyphenolic compounds entering the large intestine undergo extensive metabolism by interaction with colonic microbiota and it is metabolites and catabolites of the parent compounds that enter the circulatory system. The aim of this study was to investigate the inhibitory activity of some colonic microbiota-derived polyphenol catabolites against advanced glycation endproducts formation *in vitro* and to determine their ability, at physiological concentrations, to counteract mild oxidative stress of cultured human neuron cells.

Methods and results: This study demonstrated that ellagitannin-derived catabolites (urolithins and pyrogallol) are the most effective antiglycative agents, whereas chlorogenic acid-derived catabolites (dihydrocaffeic acid, dihydroferulic acid and feruloylglycine) were most effective in combination in protecting neuronal cells in a conservative *in vitro* experimental model.

Conclusion: Some polyphenolic catabolites, generated *in vivo* in the colon, were able *in vitro* to counteract two key features of diabetic complications, *i.e.* protein glycation and neurodegeneration. These observations could lead to a better control of these events, which are usually correlated with hyperglycemia.

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1 Introduction

Protein glycation is a spontaneous reaction depending *in vivo* on the degree and duration of hyperglycemia. Advanced

glycation endproducts (AGEs), the result of a non-enzymatic glycation reaction between amino groups of proteins and aldehydic groups of reducing sugars [1], build up slowly and

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Abbreviations: **ABTS**, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); **AGEs**, advanced glycation endproducts; **DMNQ**, 2,3-dimethoxy-1,4-naphthoquinone; **FBS**, fetal bovine serum; **MTT**, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

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can permanently alter protein structures and functions. They accumulate mainly in proteins with a long half-life, altering their structural and biochemical properties. AGEs also contribute to reducing artery, heart and lung tissue elasticity and appear to have a significant role in the progression of general cardiovascular complications associated with diabetes [2]. Moreover, these molecules have also been linked to Alzheimer's disease and neuropathy, classical features of ageing [3].

Dietary flavonoids and allied phenolic and polyphenolic compounds, including tannins and derived polyphenols, are thought to be beneficial in the control of diabetes and its complications, because of their ability to inhibit oxidative stress and protein glycation [4]. There is also growing evidence of the neuroprotective effects of phenolic compounds, most notably flavonoids, with reports on protective effects in cultured neuronal cells [5], and protection against neurodegeneration in animal models [6]. In addition, epidemiological observations have linked flavonoid intake to a reduced risk of neurodegenerative diseases [7]. Following ingestion, (poly)phenolic compounds undergo extensive metabolism during their passage through the gastrointestinal tract and, with very few exceptions, only metabolites and catabolites of the parent compounds enter the circulatory system. Metabolism occurs initially in the lumen of the small intestine with cleavage of sugar moieties after which the released aglycone undergoes glucuronidation, sulfation and/or methylation [8]. After entry of these metabolites into the circulatory system, phase II metabolism may also occur in the liver and other organs [9]. However, a large proportion of ingested polyphenols are not absorbed in the small intestine [10–12] and so pass to the large intestine, where substantial structural modifications are mediated by the colonic microflora [12–15]. The resultant catabolites, principally low-molecular-weight phenolic acids, are absorbed into the blood stream and circulate in the body prior to excretion in urine in amounts that greatly exceed those of metabolites absorbed in the small intestine, whose concentration in plasma rarely exceeds the nmol/L levels [16]. It is, therefore, of limited relevance attempting to unravel the mechanism of action of intact parent polyphenols in cell models, as these compounds rarely come into contact with tissues outside the gastrointestinal tract. Their colonic catabolites are, however, more plausible candidates for investigation of putative biological activity. The catabolites used in the current study were urolithin A and B (structures **I** and **II** in Fig. 1), which are derived from ellagic acid, a product of ellagitannin degradation following consumption of pomegranates and raspberries [17, 18], and pyrogallol (**III**), which is formed in the colon from gallic acid, an additional ellagitannin breakdown product, that can also be derived from 3-O-galloyl-flavan-3-ols [15, 18]. Other colonic catabolites include dihydrocaffeic acid (**IV**), dihydroferulic acid (**V**) and feruloylglycine (**VI**) that originate after coffee consumption [19] and 3-hydroxyphenylacetic acid (**VII**), 3,4-dihydroxyphenylacetic acid (**VIII**) and 3-methoxy-4-hydroxyphenylacetic acid (**IX**), derived from flavonoids in general, but flavonols and anthocyanins in particular, by action of the colo-

nic microbiota [10, 20, 21]. The aim of the study was to investigate the inhibitory activity of these colonic microbiota-derived polyphenol catabolites against AGEs formation and to determine their ability at physiological concentrations to counteract mild oxidative stress of cultured human neuron cells.

2 Materials and methods

2.1 Chemicals

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferrozine, glyoxal, Girard-T reagent, BSA, pyrogallol (1,2,3-trihydroxybenzene), dihydrocaffeic acid, dihydroferulic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid were purchased from Sigma-Aldrich (Milan, Italy). Feruloylglycine was a gift from Professor Takao Yokota (Teikyo University, Utsunomiya, Japan), while urolithin A and urolithin B were kindly provided by Dr. Navindra Seeram (University of Rhode Island, Kingston, RI, USA). For the oxidative stress experiment, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and DMSO were purchased from Euro-Cclone (Celbio, Italy). DMEM, fetal bovine serum (FBS), penicillin (5000 units/mL) and streptomycin (5000 µg/mL), L-glutamine (100 × solution) and trypsin (0.05%, EDTA 0.02% in PBS) were purchased from Celbio. Flasks and 96-well plates were obtained from Costar, Corning (Corning, NY). All other chemical reagents and solvents were supplied by Carlo Erba (Milan, Italy).

2.2 Albumin glycation

For the glycation experiment, BSA 40 mg/mL was incubated at 37°C for 14 days with glucose 30 mmol/L in 0.1 mol/L phosphate buffer (pH 7.4; sodium azide 0.012%) in the presence or absence of polyphenol catabolites at concentrations of 0.5, 1 and 10 µmol/L. Groups of three compounds were tested, based on the dietary source of their parent polyphenols. The "ellagitannin group", typical of pomegranate and raspberries, included pyrogallol, urolithin A, and urolithin B, the "coffee group" comprised dihydrocaffeic acid, dihydroferulic acid and feruloylglycine, and the "berry/red wine anthocyanin group" was made up of 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid. Each triplet was tested at 0.5, 1 and 2 µmol/L concentration, mimicking physiologically attainable levels in humans.

2.3 Fluorescence measurement of AGE products

Formation of AGEs was measured with fluorescence at the excitation and emission maxima of 355 and 405 nm, respectively, *versus* an incubated blank containing BSA and

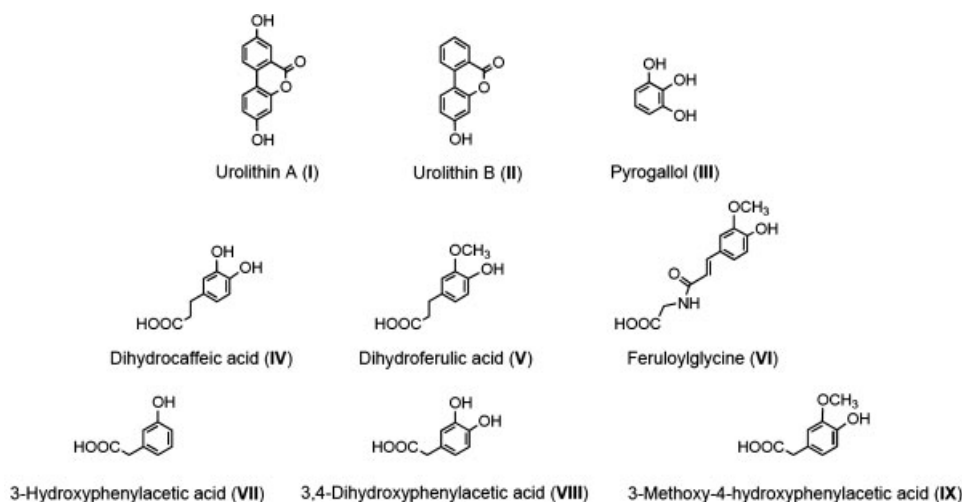


Figure 1. Structures of studied phenolic catabolites produced by degradation of dietary phenolics by the colonic microflora.

inhibitor. The percent inhibition by different concentrations of inhibitor was calculated [22].

2.4 Glucose autoxidation

Glucose 30 mmol/L in 0.1 mol/L phosphate buffer (pH 7.4, 0.012% sodium azide) was incubated at 37°C for 14 days. The degree of autoxidation of glucose was tested by measuring the formation of glyoxal with a specific spectrophotometric test that applies the Girard-T reagent [23]. Results are expressed as $\mu\text{mol/L}$ of glyoxal.

2.5 Glyoxal trapping

The dicarbonyl trapping ability of catabolites was tested by incubating for 24 h in 0.1 mol/L phosphate buffer (pH 7.4, 0.012% sodium azide) catabolites and glyoxal at concentrations of 50 μmol each (molar ratio 1:1) at 37°C. The amount of unbound glyoxal was determined with the Girard-T reagent [23] and results are expressed as % of bound glyoxal [24].

2.6 Iron chelation ability and radical-scavenging activity

The Fe^{2+} -chelation ability of catabolites was evaluated by the ferrozine assay [25]. Radical-scavenging activity was measured with the ABTS assay [26] and the results were calculated as $\mu\text{mol/L}$ of vitamin C equivalents.

2.7 Cell cultures

The human neuroblastoma SK-N-MC clonal cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cultures of SK-N-MC were prepared as described by Vettori *et al.* [27]. Cells were seeded at the

density of 40 000 cells/ cm^2 in DMEM containing 10% FBS and 50 units/mL of penicillin and 50 $\mu\text{g/mL}$ of streptomycin. Cells were maintained at 37°C in a 5% CO_2 -humidified incubator and subcultured twice a week.

2.8 Cell treatments and cytotoxicity assay

For the MTT cytotoxicity assay, exponentially growing cells were collected and suspended at a concentration of 1.2×10^5 cells/mL in fresh medium containing FBS. Thirty-six hours after seeding, cells were exposed to different concentrations of the tested polyphenol catabolites. Each compound was dissolved in DMSO. The final concentration of DMSO in contact with cells did not exceed 0.1%, which did not affect cell viability. The cells were treated with 0.1, 0.5, 1, 5, 10 and 20 $\mu\text{mol/L}$ concentrations of each catabolite for 30 min, after which they were washed with PBS before the induction of mild oxidative stress by incubation in a 2 $\mu\text{mol/L}$ solution of 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) for 24 h at 37°C after which cell viability was evaluated using the MTT assay [28]. In a second series of experiments cells were treated with groups of three compounds, based on the dietary source of their parent polyphenols. The raspberry/pomegranate “ellagitannin group” included pyrogallol and the two urolithins, the “coffee group” comprised dihydrocaffeic acid, dihydroferulic acid and feruloylglycine, and the “berry/red wine anthocyanin group” was made up of 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid. Each compound was tested at 0.5 $\mu\text{mol/L}$ for a total concentration for each group of 1.5 $\mu\text{mol/L}$.

2.9 Statistical analysis

All data are presented as the mean $\pm$ SD for three replicates for each compound or triplet of compounds. For the glycation experiments, univariate analysis of variance (ANOVA) with LSD post-hoc test was applied for multiple comparisons.

Pearson's bivariate correlation was used to correlate glycation inhibitory activity with all the other variables. The differences and correlation coefficients were considered significant with $p < 0.05$. For the neuroprotection experiment, eight spectrophotometric readings were carried out for each experimental replicate. Differences were assessed between treated and untreated cells by applying Wilcoxon's test for paired samples. A value of $p < 0.05$ was considered significant in all tests. All the statistical analysis was performed with the PASWStatistics 18.0 (SPSS, Chicago, IL, USA).

3 Results

Figure 2 shows the ability of each catabolite to inhibit protein glycation. At the lowest concentration, 0.5 $\mu\text{mol/L}$,

uroolithin A and B exhibited weak antiglycative activity that was not statistically significant, while a significant response was induced by both compounds when tested at 1 $\mu\text{mol/L}$. At the highest test concentration, 10 $\mu\text{mol/L}$, urolithin A had substantial antiglycative activity, while the effect of urolithin B was enhanced only slightly over that achieved 1 mmol/L . None of the other catabolites had a significant impact on albumin glycation with the exception of dihydroferulic acid at the highest concentration. The catabolites were also tested in groups, based on the dietary sources of the parent polyphenols, at concentrations of 0.5, 1 and 2 $\mu\text{mol/L}$. The raspberry/pomegranate group of catabolites, which comprised the two urolithins and pyrogallol, induced a significant response at the lowest concentration and inhibited protein glycation by 37 and 44% at 1 and 2 $\mu\text{mol/L}$, respectively (Fig. 3). In contrast, the "berry/red wine

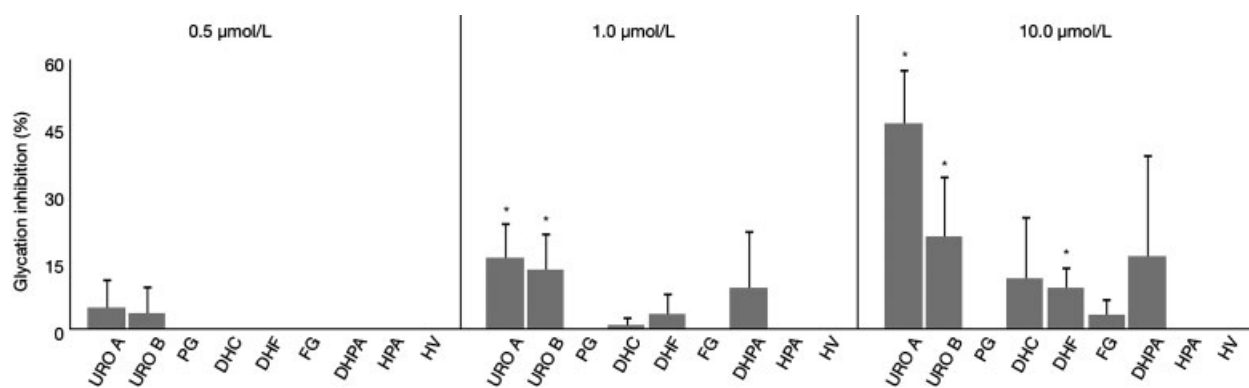


Figure 2. Percentage inhibition of BSA glycation exerted by each studied catabolite at three different concentrations. Data are expressed as the mean $\pm$ SD of three replicates. *Indicates values significantly different from untreated albumin. PG, pyrogallol; URO A, urolithin A; URO B, urolithin B; DHC, dihydrocaffeic acid; DHF, dihydroferulic acid; HPA, 3-hydroxyphenylacetic acid; DHPA, 3,4-dihydroxyphenylacetic acid; HV, 3-methoxy-4-hydroxyphenylacetic acid.

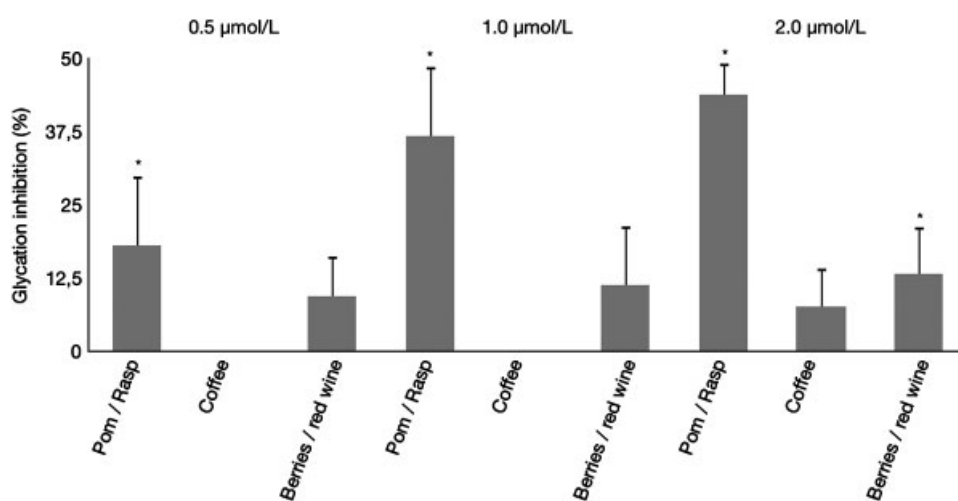


Figure 3. Percentage inhibition of BSA glycation exerted by groups of polyphenol catabolites. Pomegranate/raspberry (Pom/Rasp) = pyrogallol, urolithin A and urolithin B; Coffee = dihydrocaffeic acid, dihydroferulic acid and feruloylglycine; and Berries/red wine = 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid at 0.5, 1 and 2 $\mu\text{mol/L}$ final concentrations. Data are expressed as the mean $\pm$ SD of three replicates. *Indicates significantly different from untreated albumin.

anthocyanin" catabolites induced a significant response only at the highest test concentration while coffee catabolites were inactive. The antiglycative effects of the phenolic catabolites were not related to their antioxidant activity in the ABTS assay as urolithin A and B showed only low activity. In contrast, pyrogallol, dihydrocaffeic acid, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid, which had no significant impact on glycation, exhibited the highest antioxidant activity (Supporting Information, Fig. S1). None of the catabolites were able to bind iron also at 50 $\mu\text{mol/L}$ concentration (data not shown). The antiglycative effects of the phenolic catabolites were not related to their glyoxal-binding capacity, as DHC was the most effective in this test, followed by 3,4-dihydroxyphenylacetic acid and pyrogallol, whereas urolithin B showed a very low trapping ability (7.8%) and the remaining molecules were unable to trap glyoxal (Supporting Information, Fig. S2). Glucose autooxidation was negligible in this model, as glyoxal, a marker of glucose autooxidation, was under the detection limit of the assay after 14 days of incubation.

In the neuroprotection experiment, the selected *in vitro* model aimed to mimic oxidative stress of neuronal cells in the central nervous system. The stress was induced by DMNQ, which is commonly used to study the role of reactive oxygen species in cell toxicity, apoptosis and necrosis, and has been used to simulate oxidative stress in various cell models [29, 30]. At the low concentration of 2 $\mu\text{mol/L}$ DMNQ caused an average decrease of 40% in neuronal cell viability and was, therefore, chosen to mimic a mild physiological stress. Table 1 shows the impact of a 30 min pretreatment with phenolic catabolites, at concentrations of 0.1, 0.5, 1, 5, 10 and 20 $\mu\text{mol/L}$, on viability of human neuroblastoma SK-N-MC cells following a 24 h exposure to 2 $\mu\text{mol/L}$ DMNQ. At the two sub- $\mu\text{mol/L}$ concentrations, pyrogallol, urolithin B, dihydrocaffeic acid and 3-hydroxyphenylacetic acid exhibited significant protective effects,

with an increase in the survival of cells after induced oxidative stress ranging from 4.8 to 9.6%. At the higher concentration of 20 $\mu\text{mol/L}$, all nine compounds protected neurons against DMNQ-induced toxicity with urolithin B and dihydroferulic acid being the most potent. When assayed in combination, with each test compound at a concentration of 0.5 $\mu\text{mol/L}$, the catabolites from ellagitannins, coffee and berry/red wine anthocyanins displayed significant protection, with increased survival after oxidative stress ranging from 11 to 16% compared with cells not exposed to phenolic catabolites (Fig. 4).

4 Discussion

There is a growing realization that the colon plays an important role in the catabolism and bioavailability of dietary phenolic and polyphenolic compounds with several studies showing that even when absorbed in the small intestine, substantial quantities of polyphenols pass to the large intestine where the parent compounds are strongly modified and their catabolites can impact on both colonic health and the colonic microbiota. The level of urinary excretion indicates that substantial quantities of the colonic catabolites are absorbed and pass through the body in the circulatory system prior to excretion. Some of these compounds may play a key role in the protective effects of a fruit and vegetable-rich diet as there is evidence that they have anti-inflammatory effects in experimental models [31]. Our study investigated additional health related effects of an array of polyphenol catabolites generated *in vivo* from different food sources. In particular, we focused on their ability to counteract two features of the diabetic conditions, protein glycation and neurotoxicity linked to oxidative stress. All the tested molecules were studied at concentrations which, in view of the data of Gu *et al.* [32], could reasonably be reached *in vivo* in the context of a normal diet including polyphenol-rich food sources.

Table 1. Impact of a 30 min pretreatment with phenolic catabolites, at concentrations ranging from 0.1 to 20 $\mu\text{mol/L}$, on the survival of human neuroblastoma SK-N-MC cells subjected to mild oxidative stress by incubation for 24 h with 2.0 $\mu\text{mol/L}$ DMNQ

Phenolic catabolites (molecular weight)	Concentration ($\mu\text{mol/L}$) ^{a)}					
	0.1	0.5	1	5	10	20
Urolithin A (228)	0.5 $\pm$ 0.2	2.6 $\pm$ 2.4	3.3 $\pm$ 3.8	6.4 $\pm$ 2.5	13.8 $\pm$ 0.9 ^{b)}	11.3 $\pm$ 1.1 ^{b)}
Urolithin B (212)	4.5 $\pm$ 2.2	9.6 $\pm$ 5.0 ^{b)}	12.3 $\pm$ 3.8 ^{b)}	12.1 $\pm$ 4.0 ^{b)}	13.5 $\pm$ 1.5 ^{b)}	22.1 $\pm$ 2.8 ^{b)}
Pyrogallol (126)	4.8 $\pm$ 0.4 ^{b)}	7.1 $\pm$ 2.5	7.8 $\pm$ 3.4	8.1 $\pm$ 1.3 ^{b)}	7.9 $\pm$ 0.3	12.0 $\pm$ 2.5 ^{b)}
Dihydrocaffeic acid (182)	4.7 $\pm$ 0.3	7.8 $\pm$ 2.5 ^{b)}	7.9 $\pm$ 2.0 ^{b)}	8.3 $\pm$ 1.2 ^{b)}	8.5 $\pm$ 0.1 ^{b)}	8.4 $\pm$ 1.8 ^{b)}
Dihydroferulic acid (196)	1.0 $\pm$ 0.6	2.0 $\pm$ 1.9	5.0 $\pm$ 3.2	6.0 $\pm$ 4.0	7.0 $\pm$ 3.4	17.0 $\pm$ 4.2 ^{b)}
Feruloylglycine (251)	0.5 $\pm$ 0.5	2.4 $\pm$ 2.0	2.4 $\pm$ 2.7	2.6 $\pm$ 4.1	3.9 $\pm$ 0.8 ^{b)}	6.5 $\pm$ 0.3 ^{b)}
3-Hydroxyphenylacetic acid (152)	5.3 $\pm$ 1.6 ^{b)}	5.9 $\pm$ 0.5 ^{b)}	5.8 $\pm$ 1.5 ^{b)}	6.3 $\pm$ 0.9 ^{b)}	6.5 $\pm$ 0.2 ^{b)}	6.8 $\pm$ 1.4 ^{b)}
3,4-Dihydroxyphenylacetic acid (168)	0.6 $\pm$ 0.4	1.0 $\pm$ 1.0	3.7 $\pm$ 2.1	4.2 $\pm$ 2.8	11.0 $\pm$ 0.6 ^{b)}	11.0 $\pm$ 2.2 ^{b)}
3-Methoxy-4-hydroxyphenylacetic acid (182)	0.2 $\pm$ 0.2	3.8 $\pm$ 3.0	7.1 $\pm$ 1.8 ^{b)}	8.1 $\pm$ 0.6 ^{b)}	7.6 $\pm$ 0.4 ^{b)}	7.8 $\pm$ 1.1 ^{b)}

a) Data expressed as percentage survival $\pm$ SD ($n = 3$) relative to survival of control cells treated only with 2.0 $\mu\text{mol/L}$ of DMNQ.

b) Mean values significantly different from survival of control cells exposed only to 2.0 $\mu\text{mol/L}$ of DMNQ for 24 h ($p < 0.05$, Wilcoxon's test for paired samples).

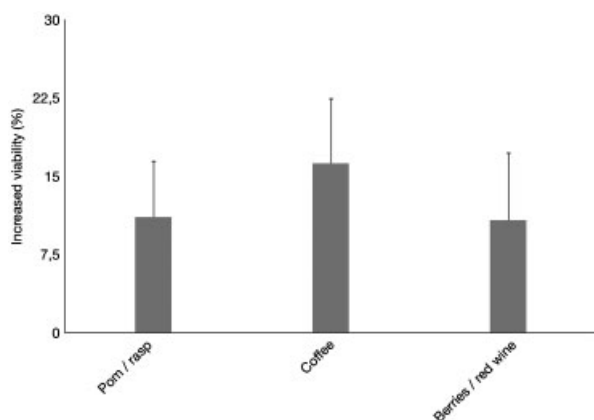


Figure 4. Impact of pretreatments with phenolic catabolites on the survival of human neuroblastoma SK-N-MC cell subjected to mild oxidative stress. Neuroblastoma SK-N-MC cells were treated for 30 min with groups of three colonic catabolites at 0.5 $\mu\text{mol/L}$ each (final concentration in the medium equal to 1.5 $\mu\text{mol/L}$ for each group) followed by a 24 h exposure to mild oxidative stress induced with 2 $\mu\text{mol/L}$ DMNQ. Pomegranate/raspberry (POM/RASP) = pyrogallol, urolithin A and urolithin B; Coffee = dihydrocaffeic acid, dihydroferulic acid and feruloylglycine; and Berries/red wine = 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid. Data are expressed as the mean increase in percentage viability $\pm$ SD ($n = 3$) compared to control cells that were not pretreated with phenolic catabolites. All three phenolic catabolite treatments exerted significant protection ($p < 0.05$).

The first evaluation was related to inhibition of protein glycation, a feature that is extremely relevant in the progression of several diabetes complications. To the best of our knowledge, this is the first demonstration that selected polyphenol catabolites exhibit antiglycative activity. In particular, urolithin A and B, generated by microbial action against ellagitannins, significantly reduced protein glycation at the physiological concentration of 1 $\mu\text{mol/L}$. Following consumption of foods containing what will invariably be a mixture of dietary flavonoids and related compounds, several phenolic catabolites will be produced in the colon and together enter the circulatory system. We, therefore, tested the effects of groups of three catabolites selected on the basis of their probable dietary origin. The group of phenolic catabolites derived from ellagitannins, the urolithins and pyrogallol, was extremely effective in protecting albumin from glycation as at a concentration of 2 $\mu\text{mol/L}$; they reduced AGE formation by almost 50% compared to untreated albumin.

The potential reactions leading to AGE formation are illustrated in Fig. 5. It has been suggested that phenolic compounds may inhibit glycation by acting as antioxidants and metal-chelating agents [33], which would involve an effect on pathway 1A in Fig. 5. This possible mechanism is, however, unlikely. The antioxidant activity of the catabolites was not correlated with their antiglycative effects and furthermore none of the catabolites, at concentrations able to inhibit glycation,

showed any chelating activity. Another possible mechanism for inhibition of glycation is a reduced dicarbonyl product-trapping ability [24], pathway 1B in Fig. 5. However, this too is unlikely as there was no correlation between the antiglycative and glyoxal trapping activities of the test compounds.

Glycation also involves glucose autooxidation pathway 2 as shown in Fig. 5 and inhibition of this route can explain the antiglycative effects [22]. However, in our model autooxidation was negligible in the presence of 30 mmol/L glucose. This leaves the glucose-mediated pathway, pathway 1 as shown in Fig. 5, as the most likely site for the inhibitory effects of the tested catabolites. In a separate study we found that coffee polyphenols were able to inhibit protein glycation and this inhibitory effect was related to a reduction of Amadori products, suggesting that phenolic compounds could act as pre-Amadori inhibitors of protein glycation [22]. Since the protein-binding ability of phenolic compounds has been reported [34, 35], it was hypothesized that polyphenols may protect albumin from glycation by a mechanism involving chemical binding [22]. This mechanism could be also relevant for the catabolites tested in the current study and this topic requires further investigation.

The second model system investigated in the current study was the protective effects exerted by the tested phenolic catabolites, at physiological concentrations, against oxidative stress induced in cultured neuronal cells. Dietary polyphenols, in particular purple grape juice and berry-derived anthocyanins, have also been shown to exert neuroprotective effects and have been associated with improved cognitive performance in animal models [36–38]. They have also been linked with protection against neurodegenerative diseases in humans [39, 40]. However, it is unclear as whether such effects are direct, or result from secondary biochemical or physiological events. In this context, there are contradictory reports as to whether or not flavonoids accumulate in the brain after consumption of foods and beverages or food extracts. Anthocyanins have been detected in animal brains in some studies [41–44], but not in others [45–48]. In the only reported human study, flavan-3-ol metabolites were detected in plasma, but not in cerebrospinal fluid, after the consumption of green tea [49].

The central nervous system is isolated from the circulation by the blood–brain-barrier, formed by the brain capillary endothelium, which selectively limits access of almost all molecules with a molecular weight of > 400 Da and $> 98\%$ of smaller molecules [50]. However, in the present study low-molecular-weight colonic catabolites of dietary polyphenols, that pass through the circulatory system before being excreted in urine, were shown to protect cultured neuroblastoma cells against mild oxidative stress. The protective effects were potentially of physiological relevance being induced by sub- $\mu\text{mol/L}$ concentrations of pyrogallol, urolithin B, dihydrocaffeic acid and 3-hydroxyphenylacetic acid, while at a dose of 20 $\mu\text{mol/L}$ protection was also observed with feruloylglycine, urolithin A and 3-methoxy-4-hydroxyphenylacetic acid (Table 1). The three combinations of different catabolites

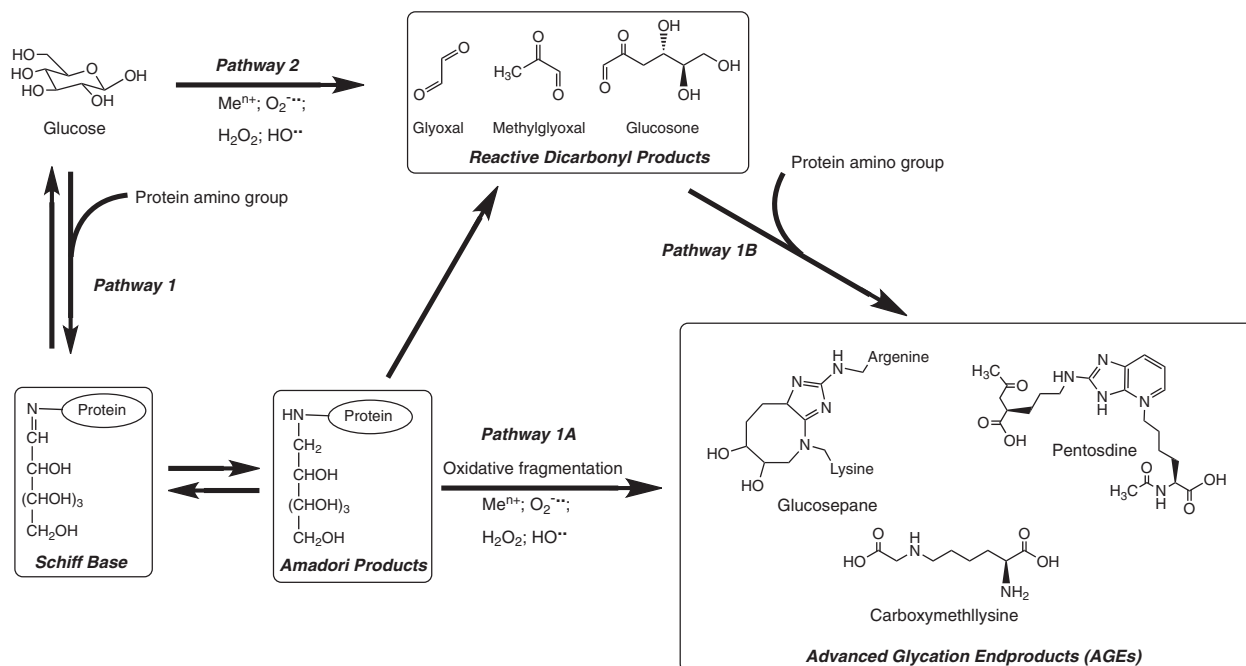


Figure 5. Possible chemical pathways leading to advanced glycation endproducts [22, 24, 33]. Me^{n+} – metal cations.

were also tested, mimicking what would be produced after the ingestion of ellagitannins in pomegranates, raspberries and/or walnuts, coffee-derived chlorogenic acids and berry and/or red wine anthocyanins. The tested concentration for each catabolite was $0.5 \mu\text{mol/L}$, and with all three combinations the vitality of the human neuroblastoma SK-N-MC cells was significantly increased (Fig. 4). The catabolites linked to coffee-derived chlorogenic acid intake, dihydrocaffeic acid, dihydroferulic acid and feruloylglycine, were the most effective, showing a 16% protection with respect to untreated cells. In general, we could rule out the possibility of an exclusively antioxidant mechanism of action of the tested molecules, as the protective effect exerted in the cultured neurons was not correlated to the antioxidant activity of the individual compound as measured with the ABTS assay.

These findings are in line with recent reports on phenolic catabolites having substantial biological activity. Larrosa *et al.* [31] investigated the anti-inflammatory effect of 18 phenolic catabolites, derived from colon microbiota, screening them by measuring prostaglandin E2 production by CCD-18 colon fibroblast cells stimulated with interleukin- 1β . They observed anti-inflammatory effects with dihydrocaffeic acid, dihydroferulic acid and 3,4-dihydroxyphenylacetic acid. González-Sarrías *et al.* [51] identified novel gene expression profiles and cellular functions modulated in Caco-2 cells in response to ellagic acid, urolithin A and B. The results suggested that at concentrations achievable in the lumen after consumption of pomegranate juice or walnuts, these ellagitannin-derived compounds might contribute to the prevention of colon cancer by modulating the expression of multiple genes in the epithelial cells lining the colon. Nevertheless, these studies

are limited to local effects in the gastrointestinal environment and the observations should not be generalized to other compartments of the human body, where different cells are present and concentrations of catabolites are undoubtedly much lower. Previous studies focussing on protective effects of phenolic components failed to test the appropriate metabolites [52], and/or reported results attributable to extremely high non-physiological concentrations of polyphenols [53]. In the current study, pyrogallol and all the hydroxycinnamates were tested in the form in which they exist in the circulatory system and, although there are marked person-to-person variations, urolithin A and B, as well as their glucuronide metabolites, have been detected in plasma after consumption of raspberries [17] and pomegranate juice/extracts [18]. Once again, all the molecules were tested at concentrations that could reasonably be expected to be reached *in vivo* in the context of a normal, fruit and vegetable-rich diet.

The current investigation demonstrated that polyphenol catabolites, generated in the colon by the action of the human local microbiota, possessed biological activity. Pomegranate/raspberry ellagitannin-derived catabolites acted as antiglycative agents, becoming good candidates in the control of cardiovascular hyperglycemia-related complications. The mechanism involved in this health related effect could be linked to binding of polyphenol catabolites to albumin, probably in specific sites that are relevant for the initiation of the glycation process, but additional experiments, such as MALDI-TOF characterization of the protein after incubation with individual components, are needed to confirm this hypothesis.

Moreover, the treatment of human neuronal cells with some phenolic catabolites protected against mild oxidative

stress in a conservative *in vitro* experimental model. The cells were exposed to the tested catabolites for only 30 min, followed by washing, so that the extracellular presence of the catabolites was precluded when treatment with DMNQ commenced. The tested compounds were applied at physiological, high nmol/L to low μ mol/L concentrations and these findings make it important to determine to what degree phenolic catabolites can pass the blood–brain-barrier and accumulate in the brain. Their low molecular weights (Table 1) suggest that they are more likely to do so than their parent compounds, many of which, in the conjugated forms that exist in the circulatory system, have a molecular weight in excess of 400 Da. It should be noted that the reduced survival following treatment of neurons with DMNQ is not necessarily mediated by free radical-induced oxidative stress, as other mechanisms including cell membrane damage, apoptosis and necrotic stimuli could be involved [29, 30].

In conclusion, some polyphenolic catabolites, known to be generated *in vivo* in the colon, were able to counteract two key features of diabetic complications *in vitro*, namely protein glycation and neurodegeneration. These observations justify more detailed investigation that could lead to a better control of the pathologies usually correlated with hyperglycemia.

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5 References

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