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ORIGINAL CONTRIBUTION

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Flavonoids inhibit the formation of the cross-linking AGE pentosidine in collagen incubated with glucose, according to their structure

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Summary *Background* Glycoxidation of collagens contributes to development of vascular complications in diabetes. *Aim of the study* Since flavonoids are potent antioxidants present in vegetal foods, it was interesting to examine their effect on the formation of a cross-linking advanced glycation endproduct, pentosidine, in collagens. *Methods* Collagen was incubated with glucose (250 mM), in the presence of different flavonoids. Pentosidine was measured by HPLC, hydroxyproline colorimetrically. *Results* Monomeric flavonoids (25 and 250 μM) markedly reduced pentosidine/hydroxyproline values in a concentration- and structure-dependent manner. In decreasing order of their specific inhibitory activity, they rank as follows: myricetin ≥ quercetin > rutin > (+)catechin > kaempferol. Thus 3′-OH or 4-oxo + Delta$_{2-3}$ increase the inhibitory activity; conjugation by Rha-Glc on 3-OH decreases it. Procyanidin oligomers from grape seed were more active than pine bark procyanidin oligomers: this may be related to the galloyl residues present in grape seed oligomers only. Procyanidin oligomers are known to be cleaved into monomers in the gastric milieu and monomeric flavonoids to be absorbed and recovered at micromolar concentrations (with a long plasmatic half-life) in extracellular fluids, in contact with collagens. *Conclusion* Flavonoids are very potent inhibitors of pentosidine formation in collagens. They are active at micromolar concentrations; these might be achieved in plasma of diabetic patients after oral intake of natural flavonoids.

Key words flavonoids – pentosidine – advanced glycation endproducts (AGEs) – collagens – procyanidin oligomers – diabetes mellitus

Introduction

Pentosidine is a specific marker of glycoxidation; this advanced glycation endproduct (AGE) cross-links peptidic chains and modifies therefore the physical properties of collagens and other proteins [26, 27]. This chemical marker is more specific than global AGE-associated fluorescence [26]. Advanced glycation or glyoxidation appears to alter glomerular permselectivity to proteins in diabetes: aminoguanidine and pyridoxamine, glycoxidation inhibitors, prevent proteinuria and retinopathy in diabetic rats [41]. Skin collagen pentosidine levels, adjusted for age, have been shown to correlate with the severity of complications in type 1 diabetic patients [37]. More

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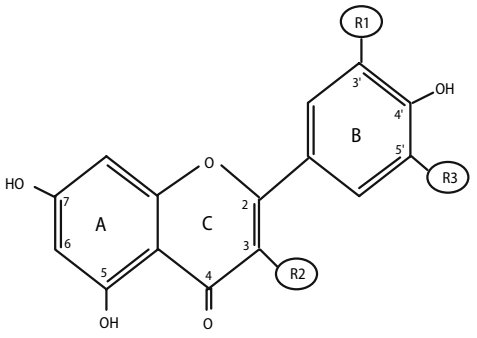
recently, when adjusted for age and diabetes duration, they were significantly associated with nephropathy and neuropathy [25]. Various antioxidants have been shown to inhibit pentosidine formation in collagen incubated with glucose [8]. This has been observed particularly with metallic ion chelators [8, 34]. Monomeric and oligomeric flavonoids are found in various plant dietary sources; their antioxidant properties have been stressed [5, 18]. We previously observed that flavonoids decreased albumin clearance and corrected hypo-albuminemia in diabetic rats [44]. It was interesting therefore to examine the effect of different flavonoids on pentosidine formation in collagen incubated with glucose and to look for the most efficient flavonoid structure. Flavonoids could indeed be useful as adjuvant nutraceutical preventive treatment of chronic complications in diabetes.

Methods

Insoluble collagen from bovine Achilles tendon (containing collagen fibres prepared by Sigma, St-Louis, MO, USA, C9879) was suspended (9 mg/ml) and incubated in 200 mM sodium phosphate buffer pH 7.4, with or without 250 mM glucose, in the presence or absence of flavonoids. After addition of 10 µl toluene, the incubation was carried out in the dark for 28 days at 37°C in a shaking water-bath, the tubes being reaerated and toluene readded every week. The collagen was then washed, lyophilized and submitted to acid hydrolysis in 6 M HCl for 20 h under vacuum, in the presence of 6 µM pyridoxamine to prevent any artificial pentosidine neoformation [26, 41]. Pentosidine was measured by HPLC [26]. Pentosidine standard was a generous gift from V. Monnier (Case Western Reserve University, Cleveland, Ohio). Hydroxyproline (Hyp) was determined colorimetrically [47]. The results were first expressed as pentosidine/Hyp ratio (pmol pentosidine/µmol Hyp; PHR). Then the specific pentosidine formation in collagen incubated with glucose (Glc) for 28 days (SPCG) was calculated for each flavonoid concentration, relative to the collagen + glucose control as follows:

[(Sample PHR with Glc) − (Sample PHR without Glc)]/[(Control PHR with Glc) − (Control PHR without Glc)]

The structures of the various flavonoids tested are given in Fig. 1. Monomeric flavonoids, aminoguanidine, EDTA and common chemicals were purchased from Sigma, DTPA, from Acros, Belgium. Monomeric flavonoids were added to the incubation medium in 20 µl ethanol to achieve 25 and 250 µM concentrations. The monomeric flavonoids remained then in solution, as observed in controls without collagen. In some cases, 2.5 µM concentration was also tested.



FLAVONOID	R1	R2	R3	4-oxo	Δ 2-3
MYRICETIN	OH	OH	OH	present	present
QUERCETIN	OH	OH	H	present	present
RUTIN	OH	Rha-Glc-	H	present	present
KAEMPFEROL	H	OH	H	present	present
(+) CATECHIN	H	OH*(S trans)*	OH	absent	absent

Fig. 1 Basic common structure and different individual functions of the monomeric flavonoids tested according to [14, 49]. Two preparations of procyanidin oligomers (PCO) were also tested. PCO from grape seeds contain (+)catechin, (-)epicatechin (C3-epimer of catechin), in oligomeric forms; these flavan-3-ols are partially esterified with gallic acid (trihydroxy-3,4,5-benzoic acid) to form 3-*O*-gallates [49]. PCO from pine bark contain only catechin and epicatechin and are devoid of gallates [42]

Procyanidin oligomers (PCO) from pine bark and from grape seed were given by N. Simonin, Laboratoire de Recherche et Développement Bourjois, Neuilly, France. Grape seed PCO contain (+)catechin and (-) epicatechin, mainly in dimeric or tetrameric forms, the oligomerisation being observed up to nonamers; the flavan-3-ols are partially esterified with gallic acid (trihydroxy-3,4,5-benzoic acid) to form 3-*O*-gallates [49]. PCO from pine bark have no measurable galloylation: they contain only catechin and epicatechin and are devoid of gallates [42]. PCO were added in 200 mM sodium phosphate buffer pH 7.4 to achieve 10 and 100 mg/dl concentrations. At high concentration PCO could precipitate in the incubation mixture. Aminoguanidine hydrochloride, DTPA and EDTA were solubilized in 200 mM sodium phosphate buffer pH 7.4 after checking and eventually adjusting the pH.

The flavonoids tested were distributed in three experiments A, B and C, with quercetin as flavonoid internal standard. Aminoguanidine was tested as positive reference advanced glycation inhibitor, EDTA and DTPA as reference chelators [34]. Each experimental condition was carried out in quadruplicate. Results are presented as mean ± SEM. In each experiment, statistical comparisons were performed by two-way analysis of variance, the two factors being glucose and treatment; this was followed by Bonferroni-Student's *t*-test. Between the experiments, comparison of internal standards was effected by one-way analysis of variance, followed by Bonferroni-Student's

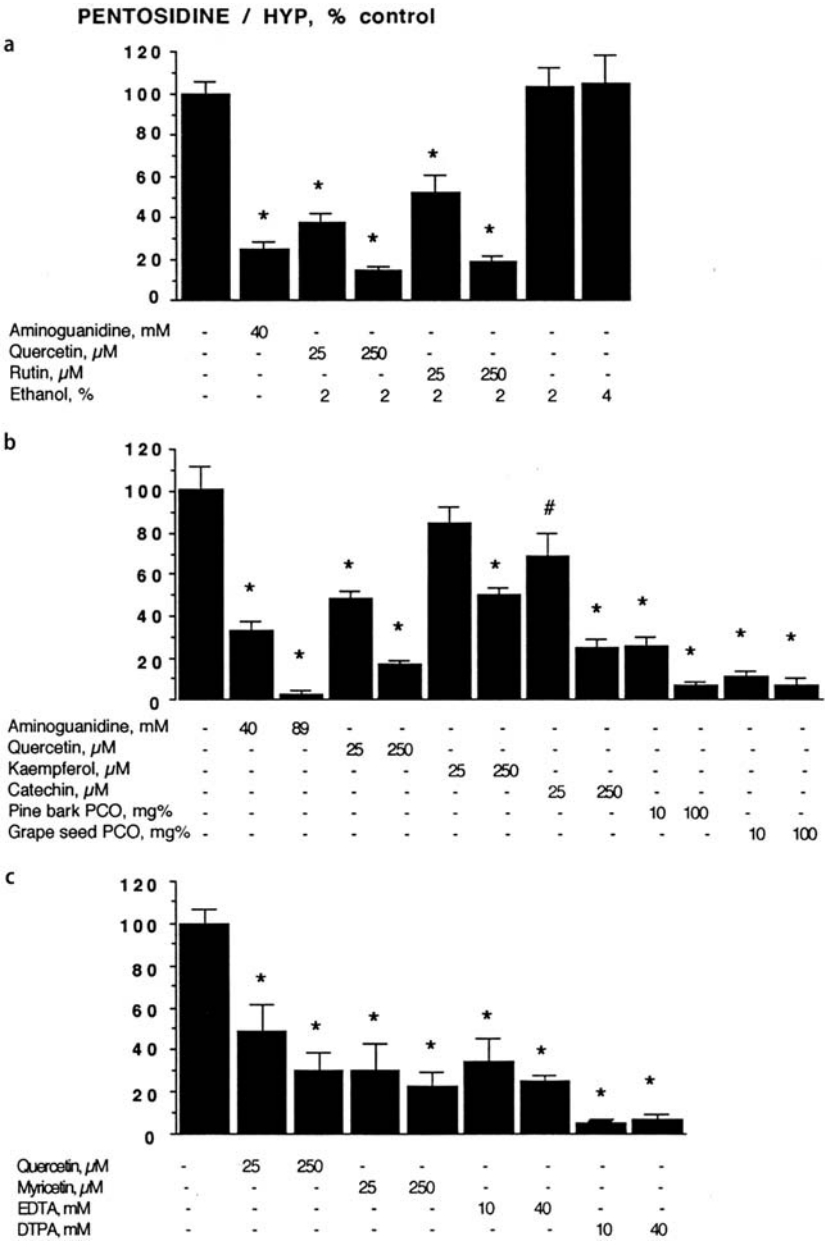


Fig. 2 Effects of aminoguanidine, monomeric or oligomeric flavonoids and EDTA or DTPA on the specific pentosidine/hydroxyproline ratio in collagen after long term incubation with glucose (SPCG), in experiments A (above), B (in the middle) and C (below). The results are expressed relative to the collagen + glucose control (CG) after subtraction of the control without glucose (see Methods). Statistical comparisons: *, $p < 0.001$; #, $p < 0.01$ vs. CG

t-test. Dose–response curves were obtained through Graph Pad Prism® software (3.02 version).

Results

Effects of quercetin and aminoguanidine on pentosidine formation

Quercetin was found to markedly inhibit pentosidine formation in experiments A, B and C (Fig. 2). At 25 μM concentration, quercetin reduced the relative specific pentosidine level in collagen (SPCG) to 37.5 ± 3.7% relative to the collagen + glucose control (CG) in experiment A ($p < 0.001$ vs. CG); to 47.8 ± 4.0% of CG in experiment B ($p < 0.001$ vs. CG; NS, A vs. B) and to 48.6 ± 11.8% of CG in experiment C ($p < 0.01$ vs. CG; NS, B vs. C). Thus the mean SPCG was 44.6% and the mean inhibition percentage 55.4%. A concentration-dependent inhibitory activity (IA) was observed for quercetin. At 250 μM concentration, quercetin reduced SPCG to 14.1 ± 1.4% of CG ($p < 0.005$, vs. 25 μM) in experiment A and to 16.8 ± 2.0% ($p < 0.005$, vs. 25 μM) in experiment B. In experiment C, a sigmoid dose–response curve was established and an apparent 50% inhibiton concen-

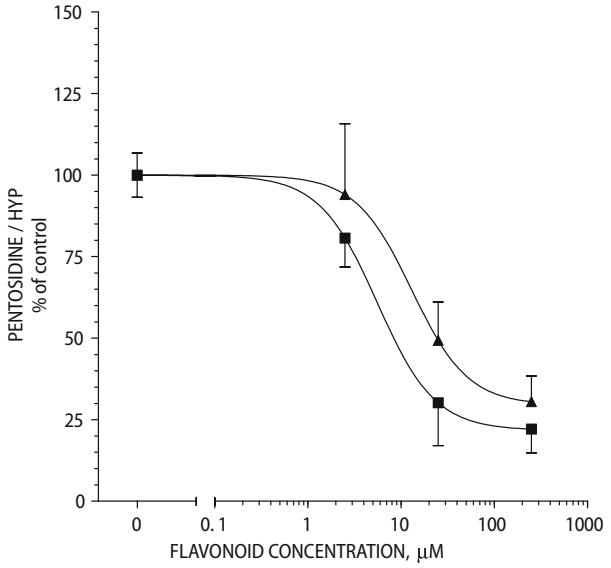


Fig. 3 Example of dose-effect curves showing the influence of quercetin (triangles) and myricetin (squares) on the specific pentosidine / hydroxyproline ratio in collagen after long term incubation with glucose (SPCG, see Methods), according to flavonoid concentration, in experiment C. The collagen + glucose control corresponds to 134.8 ± 9.2 pmol/µmol. Collagen-alone control tubes without glucose contained 8.8 ± 1.4 pmol/µmol ($p < 0{,}001$ vs. CG). The value of the collagen control without glucose was not significantly modified by the various flavonoids tested (data not shown). Myricetin $IC_{50} = 7$ µM; Quercetin $IC_{50} = 24.5$ µM

tration IC_{50} of 24.5 µM determined for quercetin (Fig. 3). Ethanol alone at 2% and 4% (v:v) did not inhibit pentosidine formation (Fig. 2).

Aminoguanidine was tested in experiments A and B: 40 mM aminoguanidine reduced SPGC to 23.7 ± 4.3% of CG in experiment A ($p < 0.001$vs. CG) and to 33.5 ± 3.3% of CG in experiment B ($p < 0.001$ vs. CG; NS, A vs. B). At 89 mM concentration, aminoguanidine reduced SPCG to 2.7 ± 1.4% of CG ($p < 0.001$ vs. 40 mM) in experiment B. The IA of 40 mM aminoguanidine was of the same order, slightly less potent, than that of 0.25 mM quercetin (SPCG of 23.7% versus 14.1%, NS, in experiment A; 33.5% vs. 16.8%, NS, in experiment B).

Effects of various monomeric flavonoids on pentosidine formation

Rutin decreased SPCG to 51.7 ± 8.6% of CG (therefore 14.2% higher than quercetin) at 25 µM concentration and to 18.8 ± 2.4% at 250 µM concentration (experiment A, Fig. 2).

(+)Catechin reduced SPCG to 68.2 ± 11.2% of CG at 25 µM concentration (20.4% higher than quercetin) and to 24.4 ± 4.5% at 250 µM. Kaempferol did not reduce significantly SPCG at 25 µM concentration (84.6 ± 7.1% of CG, therefore 36.8% higher than quercetin), but reduced it at 250 µM concentration (49.6 ± 3.7% of CG) (experiment B, Fig. 2).

In experiments A and B, a concentration-dependent IA was observed for all the monomeric flavonoids tested ($p < 0.001$, 250 µM vs. 25 µM, for rutin, kaempferol or catechin).

Myricetin reduced SPCG to 30.1 ± 13% of CG at 25 µM concentration ($p < 0.001$) and to 22.0 ± 7.1% at 250 µM concentration ($p < 0.001$) in experiment C (Fig. 2). Its dose–response curve shows an IC_{50} of 7 µM (Fig. 3).

Influence of the chemical structure of monomeric flavonoids on their inhibitory activity towards pentosidine formation (Fig. 1)

For this study, the SPCG was determined for each flavonoid at 25 µM concentration and compared with that of the quercetin standard. In decreasing order of their specific IA the monomeric flavonoids rank as follows:

myricetin ≥ quercetin>rutin>(+)catechin>kaempferol

- Conjugation by rhamnosyl-glucose decreases specific IA, with a −14.2% difference of inhibition between rutin and quercetin ($p < 0.01$);
- 3′-OH increases specific IA, with a +36.8% difference of inhibition between quercetin and kaempferol ($p < 0.001$);
- 5′-OH increases specific IA, but not significantly, with a +18.5% difference of inhibition between myricetin and quercetin ($p = 0.17$);
- 4-oxo together with $Delta_{2-3}$ increase specific IA, with a +20.4% difference between quercetin and (+)catechin ($p < 0.02$).

Effects of oligomeric flavonoids on pentosidine formation (Fig. 3)

In experiment B, pine bark PCO reduced SPCG to 25.2 ± 4.1% of CG and to 7.4 ± 1.5% of CG at 10 mg/dl and 100 mg/dl, respectively ($p < 0.001$ vs. CG; $p < 0.025$, 100 mg/dl vs. 10 mg/dl). Grape seed PCO decreased SPCG more intensively to 10.6 ± 3.0% ($p < 0.001$ vs. CG; $p < 0.01$ vs. pine bark PCO) and to 7.1 ± 3.0% ($p < 0.001$ vs. CG), at 10 mg/dl and 100 mg/dl, respectively. Therefore in decreasing order of their specific IA the PCO tested rank as follows: *grape seed PCO > pine bark PCO.*

For comparison, the monomeric flavonoid (+)catechin reduced SPCG to 24.4% of CG at 250 µM (7.2 mg/dl) concentration; if we might extrapolate on the semi-logarithmic linear curve of SPCG as a func-

Table 1 Human plasma concentration and half-life of flavonoids consumed alone or in foods

Flavonoid	Source	Quantity of flavonoid ingested (mg)	Maximum concentration in plasma (μM)	Elimination half-life (h)	Reference
Flavonols					
Quercetin	Onion	100	7.6	10.9	[10]
Quercetin-4′-*O*-glucoside	Pure compound	100	7.0	11.9	[10]
Quercetin-3-*O*-rhamnoglucoside (Rutin)	Pure compound	200	1.1	11.8	[10]
Quercetin 3-*O*-glucoside	Pure compound	156	5	18.5	[31]
Flavanols or Catechins					
Catechin	Pure compound	2000	7.8	1.1	[2]
Epicatechin	Cocoa, 26.4 g	323	5.9	NA	[16]
Epigallocatechin (EGC)	Green tea extract	115	1.2 (EGC)	1.0	[21]
			6.9 (MeEGC)	4.4	[21]
Epigallocatechin gallate	Green tea infusion, 1.2 g	88	0.33	3.4	[20]
Epigallocatechin gallate	Green tea extract	525	4.4	NA	[29]
Procyanidins					
Procyanidin dimer B2	Cocoa, 26.4 g	256	0.041	NA	[16]
Procyanidin dimer B1	Procyanidin dimer B1	18	0.011	NA	[35]

NA, not analyzed

tion of mass concentration, we would find a residual SPCG of 18% of CG for 10 mg/dl. Then the IA of catechin would be of the same order of magnitude as that of pine bark PCO (25.2%), but lower than that of grape seed PCO (7.4%). Similarly, after extrapolation, quercetin at 10 mg/dl would reduce SPCG to 13%, more efficiently than pine bark PCO and slighly less efficiently than grape seed PCO.

Effects of EDTA and DTPA on pentosidine formation

A marked reduction of pentosidine formation was observed in the presence of EDTA (with a SPCG of $34 \pm 11\%$, $p < 0.001$, and $25 \pm 3\%$, $p < 0.001$, at 10 mM and 40 mM concentration, respectively) and particularly in the presence of DTPA (5 ± 0.5 %, $p < 0.001$, and $6 \pm 3\%$, $p < 0.001$, at 10 mM and 40 mM concentration, respectively, corresponding practically to a complete inhibition) in experiment C (Fig. 2).

Discussion

The results reported here on the effects of different flavonoids and PCO on the production of pentosidine in collagen incubated with glucose, were presented in part earlier at meetings on diabetes [39, 43]. A previous study on protein glycation inhibitors extracted from thyme had reported the inhibitory activity of quercetin and eriodictyol, on global formation of AGEs (as estimated by fluorescence at 370/440 nm) in bovine serum albumin (BSA) [28]. Pentosidine however is more specific than global AGE-associated fluorescence. Besides long-lived collagens are more subject to advanced glycation than albumin in diabetes. In diabetic patients the levels of pentosidine and other AGEs [25, 40] in skin collagen were correlated with the importance of microvascular complications, whereas the pentosidine content of a plasma protein, like albumin, was not correlated [37], probably because of its much shorter half-life. More recently, the effects of several other monomeric and one dimeric flavonoids on BSA global AGEs after incubation with glucose were reported: they are in agreement with most of our own results concerning specific formation of pentosidine in collagen [17, 24, 48, 50]. Lately rutin and its metabolites containing vicinyl dihydroxyl groups (i.e. quercetin, 3–4 dihydroxytoluene and 3–4 dihydroxyphenylacetic acid) were shown to inhibit AGE formation in collagen [4].

Our results concerning the struture-dependency of the IA of monomeric flavonoids towards pentosidine formation were in accordance with the structure-dependency reported for their antioxidant properties. The latter are due to their ability to scavenge free radicals, to react with non-radicalic reactive oxygen species or/and to complex metal ions which generate them [5]. The higher the degree of OH substitution, the stronger the radical scavenging activity of a flavonoid. Additional characteristics, such as a catechol in ring B, combination of $Delta_{2-3}$ and 3-OH in ring C, appear to enhance radical scavenging activity [5, 32].

Flavonoids may be capable of binding the transition metal ions, which play a role in glycoxidation, thus preventing metal-catalysed formation of hydroxyl radicals or related species from H_2O_2 [5]. Ketol structure (4-oxo, 3-OH) in ring C and catechol in ring B appear to favour chelation. For the iron-rutin complex a 1:2 stechiometry has been suggested [5]. Under our experimental conditions it is likely that the chelating activity of flavonoids plays an important

Table 2 Examples of highest flavonoid contents of various beverages (mg/l) and foods (mg/kg fresh food, unless otherwise stated)

	Flavonols			Catechins		References
	Quercetin	Kaempferol	Myricetin	Monomers	Procyanidins	
Vegetables						
Onions	347[a]	<2[a]	<1[a]	ND[b]	ND[b]	[a][14]; [b][22]
Kale	110[a]	211[a]	<1	ND[c]	ND[c]	[a][14]; [c][11]
Broad bean	20[a]	<2[a]	26[a]	808[d]	736[d]	[a][14]; [d][6]
Broccoli	30[a]	72[a]	<1[a]	ND[c]	ND[c]	[a][14]; [c][11]
Fruits						
Strawberry	8.6[a]	12[a]	<1[a]	102[d]	103[d]	[a][14]; [d][6]
Apple	36[a]	<2[a]	<1[a]	105[e]	1000[e]	[a][14]; [e][36]
Cherry	15[a]	<2[a]	<1[a]	135[b]	700[e]	[a][14]; [b][22]; [e][36]
Cranberry	121[f]	ND[f]	142[f]	NM[g]	610[g]	[f][12]; [g][13]
Grape (black)	15[a]	<2[a]	4.5[a]	344[h]	540[h]	[a][14]; [h][46]
Grape (white)	12[a]	<2[a]	4.5[a]	ID (24%)c	ID (76%)[c]	[a][14]; [c][11]
Beverages						
Grape juice (red)	4.4[j]	<0.5[j]	6.2[j]	51[k]	99[k]	[j][15]; [k][9]
Grape juice (white)	NM[j]	NM[j]	NM[j]	18[k]	28[j]	[j][15]; [k][9]
Red wine	19[l]	2[l]	9[l]	272[e]	360[e]	[e][36]; [l][7]
White wine	<0.5[j]	<0.5[j]	1[j]	15[m]	50[h]	[h][46]; [j][15]; [m][3]
Teas (2 g/100 ml)						
Black	50[j]	32[j]	10[j]	186[d]	82[d]	[j][15]; [d][6]
Green	46[j]	30[j]	24[j]	384[d]	55[d]	[j][15]; [d][6]
Chocolate (*dark*)	NM[e]	NM[e]	NM[e]	800[e]	4300[e]	[e][36]
Cocoa (powder)*	70[n]	ND[n]	ND[n]	12000[p]	38000[p]	[n][19]; [p][16]

Variations in content are induced by various factors: plant variety, season, light and climate, degree of ripeness, food preparation & processing [1]

* mg/kg powder extract dry weight; ID, identified without quantification per weight or volume; ND, not detected; NM, not mentioned

role, since pentosidine is formed in the presence of air and trace amounts of metal ions; their trapping by EDTA or DTPA led indeed to almost complete inhibition of pentosidine formation. This is in agreement with the report that the formation of AGEs from glucose needs oxygen and trace metal ions; in contrast if glycation is induced by pentose, oxygen and trace metal ions are not necessary any more [34].

For what concerns the oligomeric flavonoids tested, grape seed PCO were significantly more effective than pine bark PCO. This may be attributed to galloylation, which is present only in grape seed PCO. Besides these grape seed PCO appeared more effective than monomeric catechin. This might be related to higher degree of polymerisation and/or galloylation of the catechin derivatives. Indeed galloylation has been shown to increase the antioxidant properties of catechins in aqueous phases; oligomerisation up to trimers also increases them, but oligomerisation from trimer to tetramer decreases them [33].

Extracellular matrix proteins, particularly collagens whose alterations play an important role in diabetic complications [25], are in direct contact with high glucose and with flavonoids present in the extracellular fluids. The IAs of 250 μM quercetin or myricetin were found here of the same order as the IA of 40 mM aminoguanidine, but at a molar concentration 60 times lower. Besides, at 25 μM concentration, quercetin and the other flavonoids tested, except kaempferol, were still significantly effective. The IC_{50} of myricetin and quercetin were found to be of 7 μM and 24.5 μM, respectively. In human subjects, plasma concentration peaks of quercetin or catechin around 7 μM after oral ingestion were reported (Table 1). Indeed flavonoids are absorbed in the intestinal tract [23] and their plasmatic half-life is remarkably long: from 10.9 h to 18.5 h for quercetin depending on the presence and the type of conjugation, and from 1 h to 4.4 h for various monomeric catechins. For what concerns PCO, their digestive absorption as such is minor: after oral ingestion, plasma peaks of procyanidin dimers B1 or B2 were found to be about 100-fold lower than those of monomeric catechins (Table 1). However in vivo effects of PCO may not require their absorption as oligomers: PCO may be cleaved in the gastric milieu into monomers, which are active and can be absorbed [23, 38].

We must be aware that the glucose 250 mM concentration of our incubation medium in vitro markedly differs from that of plasma, even in diabetic patients. However this allows to accelerate AGE formation and to increase the sensitivity of the experimental model in order to allow rapid evaluation of glycoxidation inhibitors after 1 month. In vivo glycoxidation progresses in a year-scale.

One may speculate that long-term absorption of nutraceutical flavonoids could be useful to prevent

advanced glycation of collagens in diabetic patients, in economically advanced as in developing countries. Promisingly, we observed that a chronic treatment by flavonoids decreased albumin clearance and corrected hypo-albuminemia in diabetic rats [44]. Besides, skin collagen-linked fluorescence, characteristic of AGEs, was decreased in streptozotocin-diabetic rats orally treated by rutin [30] or diosmin [45].

Various vegetal food sources contain substantial amounts of flavonoids (Table 2). Flavanols (or catechins) are major constituents of green and black tea, red wine, apples, cherries and chocolate. Flavonols such as quercetin, myricetin and kaempferol are also found in these foods and beverages, but they are predominantly present in onions, broccoli, kale, and berries (particularly in cranberry). Procyanidin oligomers are abundant in green and black tea, in red wine, but also in apples and chocolate. Nutraceutical preparations of purified flavonoids are also available, containing for instance rutin (*Esberiven*®) or grape seed PCO (*Endotelon*® or *Leucoselect*®).

In conclusion, flavonoids are very potent inhibitors of pentosidine formation in collagens. They are active at micromolar concentrations; these might be achieved in plasma of diabetic patients after oral intake of natural flavonoids.

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