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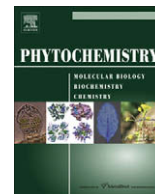
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Dihydrochalcones: Implication in resistance to oxidative stress and bioactivities against advanced glycation end-products and vasoconstriction

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ABSTRACT

Flavonoids are a group of polyphenol compounds with known antioxidant activities. Among them, dihydrochalcones are mainly found in apple leaves (*Malus domestica*). Glycosylated dihydrochalcones were previously found in large amounts in leaves of two genotypes of *Malus* with contrasting resistance to fire blight, a bacterial disease caused by *Erwinia amylovora*. In the present study we demonstrate that soluble polyphenol patterns comprised phloridzin alone or in combination with two additional dihydrochalcones, identified as sieboldin and trilobatin. Presence of sieboldin in young leaves correlated well with a high 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity. Moreover, these leaves displayed enhanced tolerance to paraquat, a photooxidative-stress generating herbicide. Interestingly, phloridzin had a high activity in the oxygen radical absorbance capacity (ORAC) assay, but its presence alone in leaves did not correlate with tolerance to paraquat. In order to further characterise the activity of these compounds, we tested their ability to prevent oxidative-dependent formation of advanced glycation end-products (AGEs) and phenylephrine-induced contraction of isolated rat mesenteric arteries. The antioxidant capacity of sieboldin was clearly demonstrated by showing that this compound (i) prevented vasoconstriction and (ii) inhibited AGEs formation. Both assays provided interesting information concerning a potential use of sieboldin as a therapeutic. Hence, our results strongly argue for a bioactivity of dihydrochalcones as functional antioxidants in the resistance of *Malus* leaves to oxidative stress. In addition, we demonstrate for the first time that sieboldin is a powerful multipotent antioxidant, effective in preventing physiopathological processes. Further work should aim at demonstrating the potential use of this compound as a therapeutic in treating free radical-involving diseases.

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1. Introduction

Numerous flavonoids are well-known potent antioxidants, effective in trapping free radicals, and may thus participate in overall plant cell redox homeostasis in plants (Rice-Evans et al., 1997; Heim et al., 2000; Hernandez et al., 2008). In apple trees, the major sub-family of flavonoids is represented by dihydrochalcones, which are found in large amounts (up to 5% of dry weight) in leaves and in immature fruits (Hunter and Hull, 1993; Treutter, 2001; Pontais et al., 2008). Although they were thought for a long time to be exclusive of *Malus* sp., dihydrochalcones have been reported

in several other genera like *Balanophora* (Tanaka et al., 2005), *Fragaria* (Hilt et al., 2003) and *Symplocos* (Ling et al., 2004). Among known dihydrochalcones, phloridzin and its aglycone, phloretin, are simple forms (Williams, 1964) and their biosynthesis in *Malus* has recently been deciphered (Judgé et al., 2008; Gosch et al., 2009). However, their function in *planta* remains unresolved. They have been hypothesised to act as UV filters in leaves (Treutter, 2006) and a role in resistance to pathogens has been suggested (Hunter, 1975; Picinelli et al., 1995; Pontais et al., 2008).

Antioxidant activity of some dihydrochalcones has been reported, but most studies on *Malus* antioxidants focused on apple fruits and not leaves (Lu and Foo, 2000; Lee et al., 2003; Khanizadeh et al., 2008; Wojdylo et al., 2008). Interestingly, phloridzin has been widely studied in the animal field notably for its effects on various diseases, notably diabetes (Ehrenkranz et al., 2005). Phloridzin was demonstrated to inhibit glucose intestinal

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absorption and renal resorption, resulting in normalisation of blood glucose and overall diminution of glycaemia in animal models (Ehrenkranz et al., 2005). Phloridzin and phloretin were also effective in preventing bone resorption in an ovariectomized rat model with chronic inflammation due to their phytoestrogenic, antioxidant and putative anti-inflammatory activities (Puel et al., 2005). Thus these data show that some of those compounds have interesting bioactivities *in vitro* and *in vivo*.

Here, we report the detection, identification, quantification and characterisation of major dihydrochalcones from leaves of two apple genotypes, using both the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and the oxygen radical absorbance capacity (ORAC) *in vitro* assays. Two additional tests relying on ROS-dependent mechanisms, i.e. inhibition of the formation of advanced glycation end-products (AGEs) and antioxidant-dependant

vasorelaxant activity on isolated rat mesenteric arteries were performed to validate the antioxidant bioactivities. Because AGEs and hypertension participate, among other pathologies, in complications linked to diabetes, like atherosclerosis and nephropathy (Singh et al., 2001), both assays also provided information concerning a potential therapeutic use of these molecules. Phloridzin was detected in both *Malus* genotypes, whereas trilobatin and sieboldin were found in the leaves of only one of the genotypes. We found that sieboldin was very efficient in the DPPH radical scavenging assay and that its presence in apple leaves correlated well with tolerance to photooxidative stress. Sieboldin was also very active in inhibiting AGE formation and preventing phenylephrine-induced contraction of isolated mesenteric artery segments. Hence, we (i) provide evidence for the complementary activities of sieboldin and phloridzin in tolerance to oxidative stress in *Malus* leaves

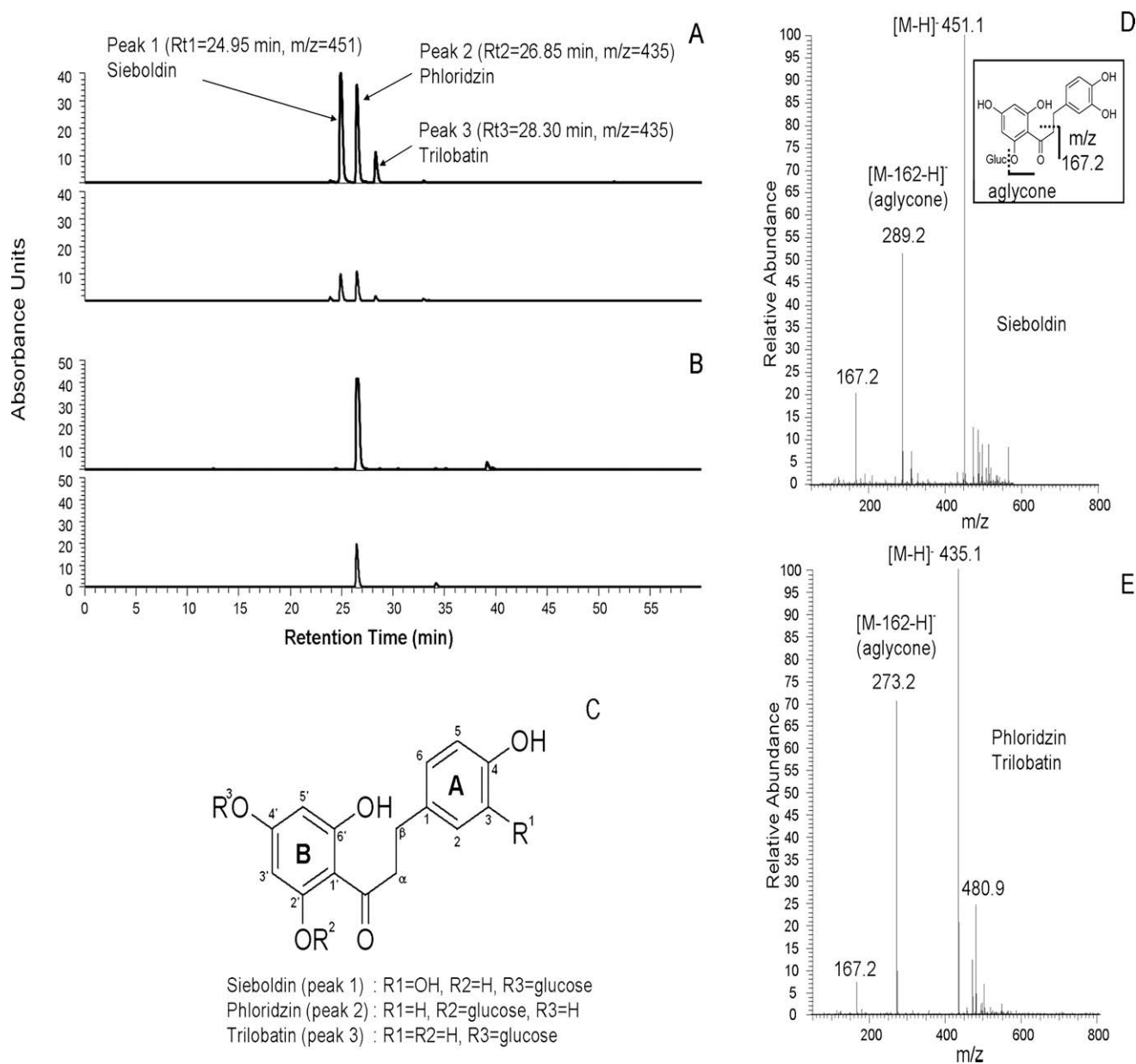


Fig. 1. UV-chromatogram at 280 nm of extracts of young (black) and mature (grey) leaves from Evereste (A) and MM106 (B) separated by reversed-phase HPLC. Retention times, m/z (M-H) and chemical structures obtained from NMR spectra are given for each compound. (C) Chemical structures of compounds 1, 2 and 3. (D) and (E) mass spectra of sieboldin (D) and phloridzin and trilobatin (E) which are identical for those two compounds. A typical fragmentation of sieboldin in the negative mode is represented in panel D, this fragmentation is similar for the two other compounds.

and (ii) demonstrate that sieboldin should be considered for further investigation on animal models concerning potential anti-AGE and anti-hypertensive activities.

2. Results and discussion

2.1. Leaf flavonoid profiles

Our initial aim was to identify phenolic compounds in leaves of two genotypes (MM106 and Evereste) displaying contrasted susceptibilities to fire blight. As previously described in the literature (Hunter and Hull, 1993; Treutter, 2001; Pontais et al., 2008), *Malus* young leaves contained high levels of dihydrochalcones (Figs. 1 and 2). Polyphenol leaf patterns of both genotypes predominantly displayed either one or three major peaks (Fig. 1A and 1B). Peak 2 retention time (R_t = 26.85 min) coincides with an authentic standard of phloridzin which was confirmed with its mass spectrum giving a quasi-molecular ion $[M-H]^-$ at m/z = 435 and a fragment ion at m/z = 273 corresponding to the loss of the glucose moiety ($[M-163]^-$; Fig. 1E). This compound was common to both genotypes. Evereste leaves displayed two other peaks (Fig. 1A). Peak 1 with R_t = 24.95 min and with a pseudo-molecular ion $[M-H]^-$ at m/z = 451 could be attributed to a hydroxylated form of phloridzin (Fig. 1D). Peak 3 with R_t = 28.30 min and with $[M-H]^-$ at m/z = 435, the same pseudo-molecular ion than for phloridzin, was attributed to an isomer of phloridzin (Fig. 1E). A minor peak at R_t = 23.93 min was also observed and showed an $[M-H]^-$ ion at m/z = 289 which is in accordance with a hydroxylated form of phloretin. Other very minor compounds were also detected showing pseudo-molecular ions in accordance to the loss of two hydrogen atoms in comparison with the m/z of major compounds. This suggested that they probably were oxidation products of the major compound studied or other chalcones. However, unlike Pontais et al. (2008) who detected phloretin in lyophilized leaves, phloretin was only detected as trace in the present study. This can probably be explained by the fact that we used fresh leaves for the analysis, preventing the ectopic conversion of phloridzin to its aglycone phloretin. As in the present study, we had previously identified other flavonoids like flavanols but in very small amounts (Pontais et al., 2008). This is in agreement with the fact that they are preferentially accumulated in mature apple fruits (Khanizadeh et al., 2008; Wojdylo et al., 2008).

Compounds 1, 2 and 3 were then isolated as described in the Section 4 and the yield was around 60% for each compound (600 mg of purified sieboldin from 10 g of leaf dry weight). Their chemical structures were determined by means of spectroscopic analysis including 1D and 2D 1H and ^{13}C NMR, using the assignments of previously identified dihydrochalcones (Hilt et al., 2003; Ling et al., 2004). As described above, 2 exhibited a pseudo-molecular ion $[M-H]^-$ at m/z = 435 which could be associated to the molecular formula $C_{21}H_{24}O_{10}$. It was then readily identified as phloridzin through 1H and ^{13}C NMR analysis and comparison with literature data (Hilt et al., 2003). Compound 3 shared the same molecular formula with 2 but its 1H NMR spectrum clearly revealed a different substitution pattern at ring A of the aglycon moiety (phloretin). Indeed H-5' and H-3' appeared as a singlet (2H), pointing to an *O*-glycosylation at C-4'. This was confirmed after close inspection of the ^{13}C NMR spectrum of 3 (Table 1) together with extensive HMBC correlation analysis. Therefore 3 was identified as trilobatin or phloretin-4'-*O*-glucoside (Ling et al., 2004). By comparison to 2 and 3, the pseudo-molecular ion $[M-H]^-$ of compound 1 was shifted up to m/z = 451 (+16 u) suggesting the presence of an additional hydroxyl group in the molecule. In the 1H NMR spectrum of 1, the aromatic protons of ring A were characterised as an ABX system of spins at δ_H 6.39 (1H, d, J = 2.0 Hz), 6.33 (1H, d, J = 8.5 Hz) and 6.51 ppm (1H, dd, J = 8.5, 2.0 Hz), respectively. Peak 1 was thus supposed to be the 3-OH derivative of trilobatin (3) i.e. sieboldin or 3-hydroxyphloretin-4'-*O*-glucoside (Ling et al., 2004), and again this was confirmed through ^{13}C NMR (Table 1) and HMBC correlation analysis. All chemical structures are indicated on Fig. 1C. The phloridzin biosynthetic pathway has recently been deciphered (Judgé et al., 2008; Gosch et al., 2009) but the detection of sieboldin and trilobatin at high constitutive levels raises the possibility for new enzymatic steps in *Malus*. Trilobatin may originate from the isomerisation of phloridzin or specific glucosylation of phloretin and sieboldin from cresolase-catalysed oxidation of the trilobatin B-ring. Alternatively, sieboldin may be formed after chalcone synthase-catalysed extension of caffeic acid, with subsequent 4'-*O*-glucosylation.

Although phenolic compositions were qualitatively very similar between the two leaf stages in a same genotype, dihydrochalcones in mature leaves were quantitatively somewhat different from young leaves (Figs. 1 and 2). Indeed, total soluble polyphenol content in younger leaves, calculated as the sum of all compounds

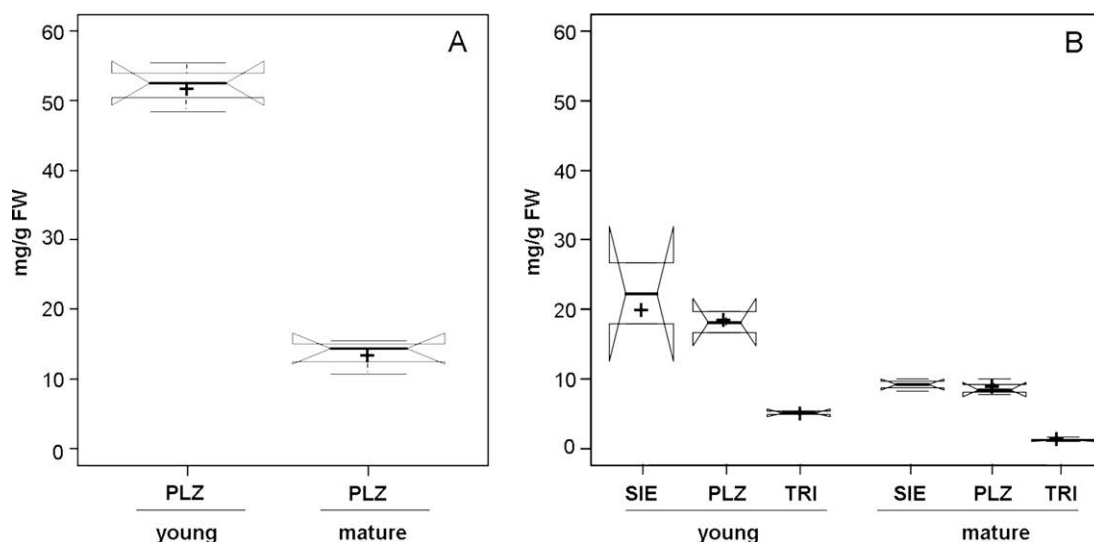


Fig. 2. Quantification of major dihydrochalcones (sieboldin (SIE), phloridzin (PLZ) and trilobatin (TRI)) from MM106 (A) and Evereste (B) young and mature leaves. Concentrations are expressed in mg per gram of fresh weight (FW). Data from three independent biological measurements. For interpretation of box-and-whisker plots, see Section 4.

Table 1
¹³C NMR (125 MHz) assignments for **1** and **3** in CD₃OD.

Attribution	Compound 1	Compound 3
Ring A		
1	134.6	133.9
2	116.5	130.2
3	146.1 (OH)	116.2
4	144.3 (OH)	156.4 (OH)
5	116.3	116.2
6	120.6	130.2
α	47.5	47.3
β	31.4	31.2
CO	207.0	207.2
Ring B		
1'	106.8	107.1
2'	165.4	165.3
3'	96.4	96.6
4'	165.0	164.9
5'	96.4	96.6
6'	165.4	165.3
Ring C		
1''	101.1	101.3
2''	74.6	74.7
3''	78.3	78.3
4''	71.1	71.3
5''	77.9	78.1
6''	62.3	62.5

Assignments were based on HMQC and HMBC experiments.

isolated (for detail see Section 4), was higher than that of mature leaves, in particular in MM106 for which young leaves contained as much as three times more phloridzin than in mature ones (Fig. 2). In addition, the quantification of each compound confirmed that there is no major difference in total dihydrochalcone content between the two genotypes, as previously shown (Pontais et al., 2008). Phloridzin represents more than 95% of the dihydrochalcones present in MM106 leaves, but in Evereste the same concentration of dihydrochalcones is distributed into three compounds, sieboldin, phloridzin and trilobatin (Fig. 2). Both trilobatin and sieboldin were identified in other *Malus* species in previous studies (Williams, 1964; Hunter, 1975). However, their antioxidant capacities and bioactivities have never been investigated so far. The DPPH scavenging capacity, which evaluates general radical antioxidant activity (Sharma and Baht, 2009), and the ORAC assays, which tests hydrophilic chain-breaking antioxidant capacity against peroxy radicals (Ou et al., 2001), were thus performed on pure compounds.

2.2. Antioxidant activities of purified dihydrochalcones isolated from leaves

In order to determine if the soluble polyphenols may participate significantly to overall antioxidant potential, we performed both DPPH scavenging and ORAC assays on purified compounds, as shown in Fig. 3. We also included phloretin, the aglycone of phloridzin, to determine which pharmacophores may influence antioxidant potential. The two assays gave different results. Sieboldin appeared to be the most efficient dihydrochalcone in reducing DPPH, both through a high Trolox equivalent activity (3 μmol of Trolox per μmol of sieboldin, i.e. more than 10-fold that of other dihydrochalcones) and almost immediate reduction of DPPH. Phloridzin displayed the weakest activity with this assay and no significant difference was observed with phloretin or trilobatin. Nevertheless, both were twofold more active than phloridzin. In addition, the kinetics of DPPH reduction by these compounds was slow as they reduced less than 50% of initial DPPH within 40 min. By contrast, phloridzin was the strongest tested dihydrochalcone for the ORAC assay (15 μmol of Trolox/μmol of

compound) and phloretin the weaker (5 μmol of Trolox/μmol of compound). Sieboldin and trilobatin were intermediates between these two other dihydrochalcones (10 μmol of Trolox/μmol of compound). Two other well-known plant antioxidants were tested as references, under the same conditions, glutathione and ascorbic acid (Noctor and Foyer, 1998). These two antioxidants displayed a low activity in both assays. This is probably due to the fact that these two compounds are not regenerated *in vitro*, unlike *in vivo* where their regeneration is achieved by specific enzymes, i.e. dehydroascorbate reductase, monodehydroascorbate and glutathione reductase (Hernandez et al., 2008). However, the kinetic of DPPH reduction by ascorbic acid was similar to sieboldin. Dihydrochalcones from *Malus* leaves may thus be classified differentially depending on the antioxidant assay: phloridzin > sieboldin > trilobatin > phloretin for the ORAC assay and sieboldin > phloretin = trilobatin = phloridzin for the DPPH assay. In a recent study, diverse dihydrochalcones were shown to have different DPPH radical scavenging capacities and to inhibit lipid peroxidation more efficiently than their flavanone analogs (Nakamura et al., 2003). Furthermore, phloridzin was shown to have a relatively low antioxidant activity in comparison with other apple polyphenols but was however a little more efficient than vitamin C in scavenging superoxide anion (Lu and Foo, 2000). These data are in accordance with our results showing that certain dihydrochalcones, such as phloridzin and sieboldin, have a high and somewhat specific antioxidant potential.

2.3. Antioxidant activities of leaves of the two *Malus* genotypes

2.3.1. Antioxidant activities of leaf extracts

Antioxidant potential determined by both the ORAC and DPPH radical scavenging assays for two leaf ages (young and mature) and both genotypes are shown in Fig. 4. In the DPPH radical scavenging assay, Evereste young leaf extracts displayed the highest activity which was 5-fold higher than that from MM106 extracts, which had a very weak activity. Evereste mature leaf extract activity in the DPPH assay was intermediate. Different results were obtained with the ORAC assay. Indeed, young and mature Evereste leaf extracts and MM106 mature leaf extracts exhibited similar values (500 μmol of Trolox per gram of fresh weight), which were much weaker than those of MM106 young leaves (more than 1000 μmol of Trolox per gram of fresh weight).

Although the sum of antioxidant activity of individual compounds does not perfectly match the total antioxidant activity in leaves (Figs. 3D and 4), a strong correlation exists between antioxidant activity of each compound, leaf content and measured total leaf antioxidant activity. Indeed, theoretical calculations revealed that the major dihydrochalcones isolated represented up to 70–140% of total measured antioxidant activity in young leaves with the two tests (Fig. 3D). For example, total activity calculations based on major dihydrochalcone concentrations in Evereste young leaves and antioxidant activity gave a DPPH radical scavenging activity of 170 μmol of Trolox per gram of fresh weight, whereas the experimentally measured activity of extracts from Evereste young leaves was 250 μmol of Trolox per gram of fresh weight with this test. Phloridzin is in very high amounts in MM106 and participates in whole leaves oxygen radical absorbance capacity, as shown in Fig. 3D, calculated antioxidant activity and experimentally measured activity being similar (1700 μmol of Trolox per gram of fresh weight). In addition, Evereste leaves contain smaller amounts of phloridzin and two other dihydrochalcones, trilobatin and sieboldin, the latter displaying a very strong activity in the DPPH assay. Its presence is strongly correlated with leaf extract total antioxidant capacity measured with this assay. Furthermore, lower amounts of phloridzin in Evereste young leaves correlated well with the weaker ORAC values than those determined in

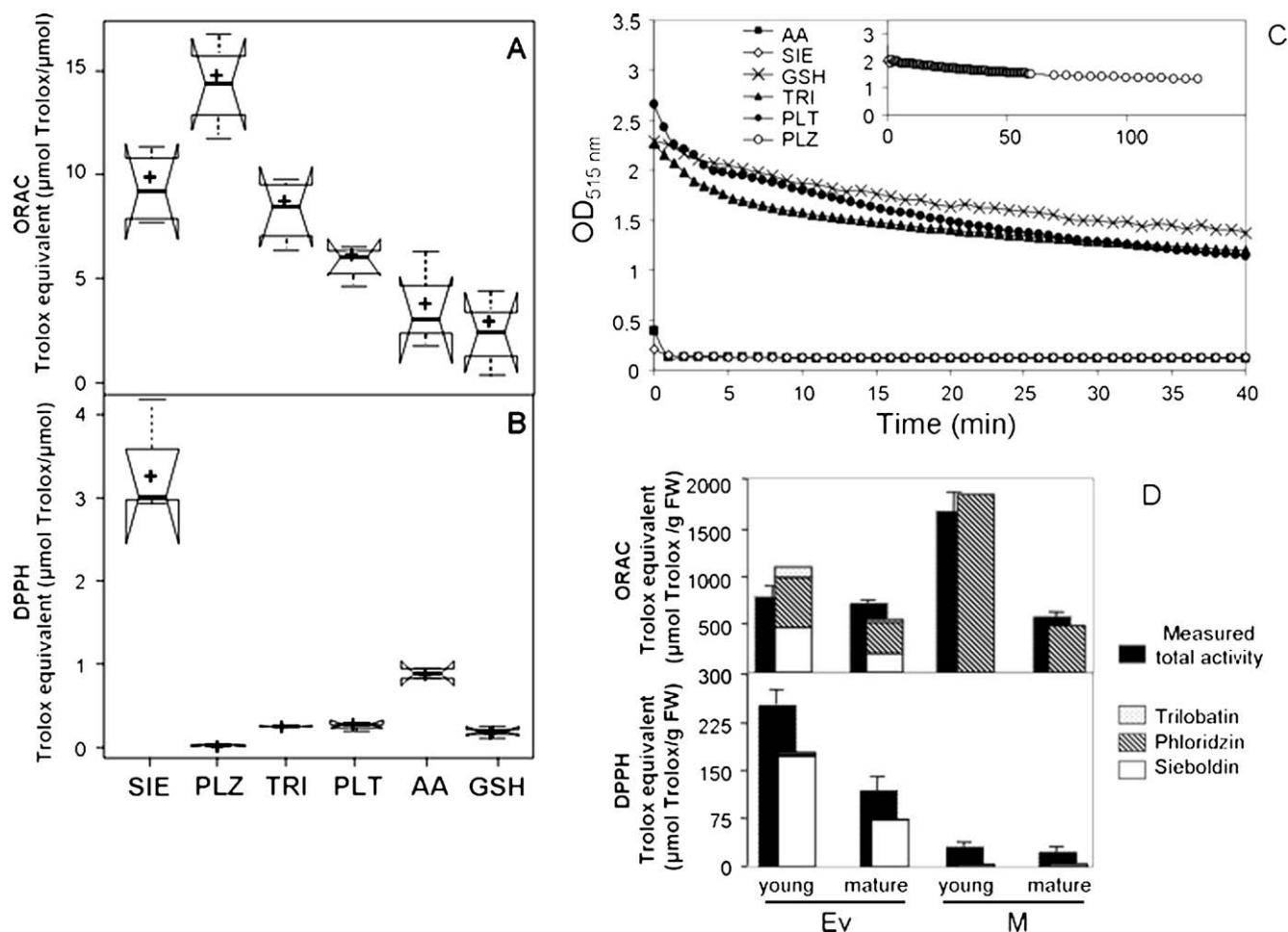


Fig. 3. ORAC and DPPH scavenging activity of sieboldin (SIE), phloridzin (PLZ), trilobatin (TRI), phloretin (PLT) and reference compounds, ascorbic acid (AA) and glutathione (GSH). Measured ORAC using compound concentrations ranging from 62.5 μ M to 1 mM (panel A) and DPPH scavenging activity and kinetics using concentrations ranging from 6.25 μ M to 3 mM (panels B and C) of individual compounds expressed in μ mol of Trolox/ μ mol of compound. Panel D, calculated contribution to whole leaf antioxidant activity, in μ mol of Trolox/g of fresh weight (FW), calculated as the product of the leaf compound concentration (Fig. 2) by its antioxidant activity and molecular weight. Data from Fig. 4 are also represented in panel D (black histograms) for comparison.

MM106. However, it should be noted that total antioxidant activity in leaves may also include other enzymatic antioxidants such as superoxide dismutase, catalase and peroxidase.

2.3.2. Protection against paraquat-induced oxidative stress

Paraquat is an herbicide known to be a potent photooxidant that blocks photosystem I and enhances ROS production leading to cell death (Donahue et al., 1997). The effect of paraquat-derived oxidative stress may be monitored *in vitro* by measuring the conductivity of the bathing media surrounding paraquat-infiltrated leaf discs. We hypothesised that a high constitutive antioxidant capacity may prevent or block this oxidative cell death. As the young leaves of both Evereste and MM106 have specific polyphenol compositions (Fig. 1) and antioxidant capacities (Fig. 4), leaf discs from both genotypes may display different susceptibilities to the herbicide. The conductivity of the incubating medium containing Evereste and MM106 young leaf discs pre-infiltrated with paraquat is shown in Fig. 5. The 10 discs used for this purpose represented about 2.5 mg of total dihydrochalcones in both genotypes, i.e. about 6 μ mol of phloridzin equivalent (based on the data from Fig. 2). Electrolyte leakage from MM106 leaf tissues infiltrated with paraquat increased faster than that of Evereste, for the highest concentrations tested (10 and 100 μ M). Maximal electrolyte leakage of MM106 leaf tissues was obtained in less than 5 and 15 h for paraquat at 100 and 10 μ M, respectively, versus 12

and 20 h for Evereste at the same concentrations. The lower concentration of paraquat (1 μ M) was ineffective on Evereste leaf discs while it still induced a progressive increase in electrolyte leakage from MM106 tissues (Fig. 5). As a consequence, Evereste young leaves are less sensitive to paraquat-derived oxidative stress than those of MM106. These results clearly show a strong difference in the constitutive antioxidant potential of both genotypes.

The high DPPH scavenging ability of young Evereste leaves, together with the presence of sieboldin, correlates well with tolerance to photooxidative stress, whereas high ORAC activity of MM106 and the presence of high amounts of phloridzin did not. Tabart et al. (2009) have recently shown that different antioxidant assays are not necessarily correlated for the same compound. In our study, results obtained for sieboldin and phloridzin clearly illustrate this and they highlight the necessity to use several antioxidant assays and even then the interpretation regarding the biological significance of the measured activity remains limited (Huang et al., 2005). However, in our case, the values obtained with the DPPH assay correlated strongly with those of the paraquat bioassay, which involves superoxide anion scavenging (Donahue et al., 1997).

In order to further characterise the two dihydrochalcones with the highest antioxidant potential, i.e. sieboldin and phloridzin, and test the significance of our results obtained with both the ORAC and DPPH assays, we tested the ability of phloridzin and sieboldin

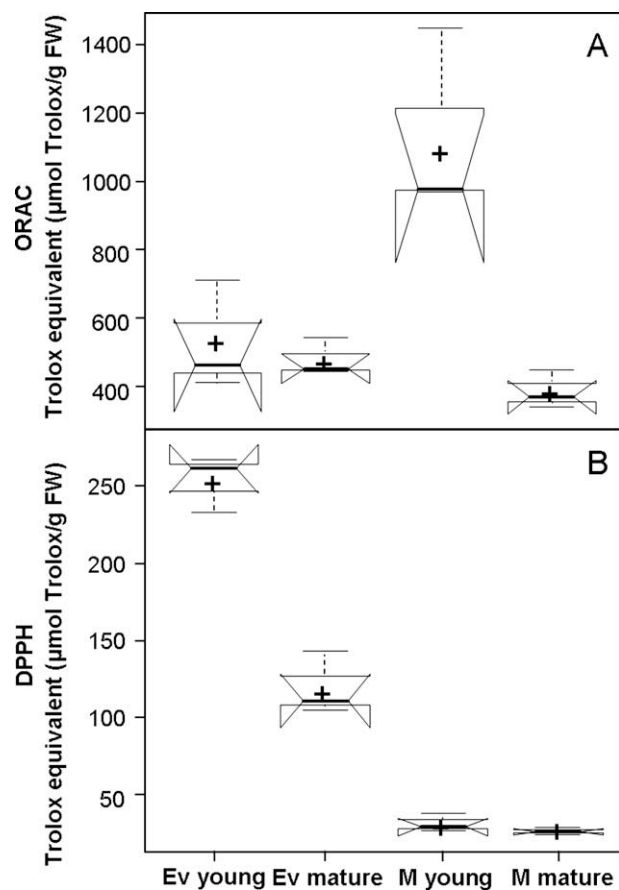


Fig. 4. Box-and-whisker plots of antioxidant capacities of methanolic extracts of young and mature leaf extracts (initial concentrations were 150 mg of fresh weight per ml) of Evereste (Ev) and MM106 (M) *Malus* genotypes assayed by the ORAC (A) and DPPH scavenging assays (B). Data represent duplicates from three independent experiments and are expressed in μmol of Trolox per g of fresh weight (FW).

(i) to antagonize phenylephrine-induced vasoconstriction on isolated rat mesenteric arteries and (ii) to inhibit the Maillard reaction leading to the formation of advanced glycation end-products.

2.4. Dihydrochalcone bioactivity

2.4.1. Vasorelaxant activity on isolated rat mesenteric arteries

The resistance of arteries controls the blood flow to the tissues through their ability to adapt their diameter in response to pressure and flow. Artery contraction may be achieved through NADPH oxidase-dependent formation of superoxide anion (Luscher et al., 1992; Alvarez et al., 2008). This superoxide anion-induced constriction may thus be prevented by biologically active antioxidant compounds. We therefore used phenylephrine on isolated rat mesenteric artery segments to provoke superoxide anion-dependant and endothelium-independent constriction of smooth muscle cells, and test the ability of isolated dihydrochalcones to counteract this process (Alvarez et al., 2008). This assay was previously used to demonstrate the vasorelaxant properties of green tea derivatives of catechin (Huang et al., 1998). As shown in Fig. 6, 5×10^{-5} M phenylephrine induced contraction of rat mesenteric arteries (mock). This contraction could not be prevented by a preincubation with 10^{-5} M phloridzin but a 50% decrease in contraction was obtained with preincubation with sieboldin at the same concentration (corresponding to a final quantity of 50 nmol in the vessel, a quantity found in approximately 1 mg of fresh leaf tissue). Both apocynin (a NADPH oxidase inhibitor) and tempol (a superoxide dismutase

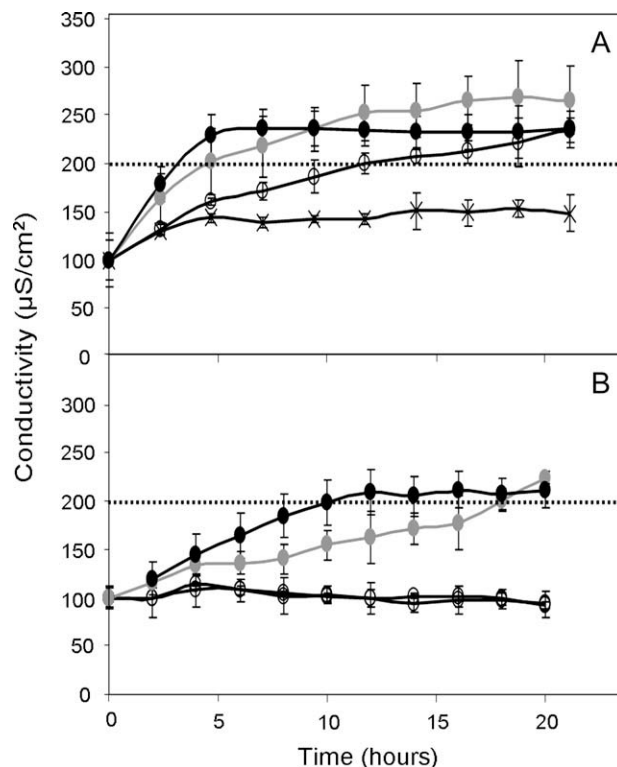


Fig. 5. Conductivity (μS/cm²) of bathing solutions containing leaf discs of MM106 (A) and Evereste (B) pre-infiltrated with paraquat at 1 (open circles), 10 (grey) and 100 (black) μM or assay medium (crosses) under constant illumination (means of triplicates from one typical experiment). The dot line at 200 μS/cm² was drawn for comparative purposes between the two apple genotypes.

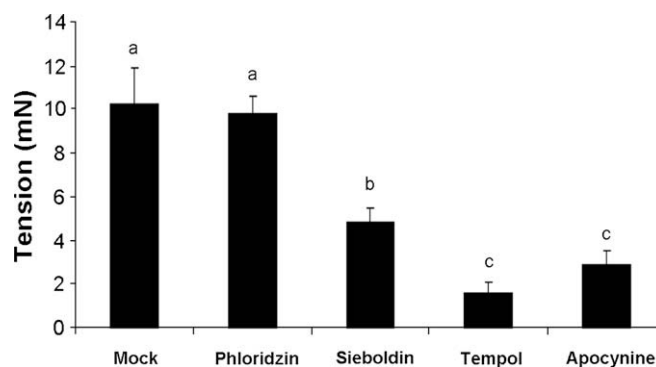


Fig. 6. Contraction of isolated mesenteric arteries in response to 5×10^{-5} M phenylephrine, with 30 min preincubation in 10^{-5} M phloridzin, sieboldin or control vasorelaxants, tempol and apocynin ($n = 3$, means from three independent artery segments). Mock indicates the result of artery contraction before preincubation. Letters indicate statistical classes for both assays ($p < 0.01$).

mimetic, Wilcox and Pearlman, 2008) were more efficient than the two dihydrochalcones. Cumulative concentration–response curves with sieboldin and phloridzin on pre-contracted arteries (phenylephrine 10^{-6} M) did not show any effect of these compounds (data not shown). Although activation of NO synthase or other relaxing factors cannot be excluded, the strong *in vitro* radical scavenging activity of sieboldin (Fig. 5) suggests it can efficiently trap superoxide anion in an *ex vivo* model. However, as phloridzin is rapidly converted to its aglycone *in vivo* and excreted in the urine (Ehrenkranz et al., 2005), the concentration of sieboldin used here might be relevant *in vivo* in prolonged administration only. Sesamin, a lignan found in sesame seeds and

Table 2

Inhibitory concentration for 50% formation of AGEs. Aminoguanidine was used as a reference compound.

Compound	IC ₅₀ (mM)
Aminoguanidine	8.5
Phloridzin	2.5
Sieboldin	0.2
Ascorbic acid	NA
Glutathione	NA
Trolox	1.7

NA means no detectable activity.

oil, is active *in vivo* in preventing hypertension. It is rapidly converted to other metabolites in liver and plasma concentrations of sesamin are about 0.2 $\mu\text{mol/l}$ (Nakano et al., 2006). At this concentration, sieboldin might not be active enough to produce physiological effects, but its action would be greatly dependent on its bioavailability and metabolism.

2.4.2. Inhibition of the formation of advanced glycation end products (AGEs)

A protocol adapted from Vinson and Howard (1996) was used to test the capacity of phloridzin and sieboldin to inhibit AGE formation, in comparison to standard antioxidants ascorbic acid, glutathione, Trolox and anti-AGE aminoguanidine. The Maillard reaction may occur between a reducing sugar and an amino-compound and lead to the formation of N-substituted glycosylamine (Zhang et al., 2009). Amadori products are then formed through intramolecular rearrangement and dehydration. ROS-mediated oxidative degradation of Amadori products results in the formation of carbonyl and dicarbonyl compounds known as intermediate glycation products (IGP) and carboxymethyl-amines (e.g. N-carboxymethyllysine), also considered as AGEs. Subsequent chain-reactions may lead to intra- and inter-protein cross-linking, resulting in physiological disorders (Goldin et al., 2006). AGEs have been correlated with many complications associated with diabetes because hyperglycaemia enhances protein glycation and are also responsible for increased oxidative stress (Singh et al., 2001; Reddy and Beyaz, 2006). Our results (Table 2) indicate that, in comparison with the reference compound aminoguanidine which entered the phase II clinical trials but was withdrawn because of its side effects (Reddy and Beyaz, 2006), the two dihydrochalcones were clearly more efficient in inhibiting the formation of AGEs, since their IC₅₀ was much lower. Sieboldin displayed a 40-fold lower IC₅₀ (0.2 mM) than aminoguanidine and a 10-fold lower IC₅₀ than phloridzin (Table 2). This concentration is found in about 5 g of fresh leaf tissue. Anti-AGE properties are linked to restriction of the Maillard reaction, by direct interference with either the sugar or the protein, or through ROS scavenging activities blocking the oxidative degradation of Amadori products, or through carbonyl and dicarbonyl compound neutralization by conjugation. AGEs may also be degraded by crosslink breakers (Reddy and Beyaz, 2006). Our AGE inhibition test did not allow us to determine the precise mechanism by which phloridzin and sieboldin are effective against AGEs, but a recent study reports that phloridzin and phloretin are able to quench glyoxal and methylglyoxal, two dicarbonyl species (Shao et al., 2008). Sieboldin, being several fold more efficient than phloridzin in inhibiting AGE, and in view of the results of the antioxidant assays, its high radical scavenging activity may also participate in inhibiting the formation of IGPs.

3. Conclusion

In conclusion, our data suggest that dihydrochalcones are involved in the overall antioxidant potential of *Malus* leaves. In addition to the demonstration of sieboldin bioactivity, our results

highlight the potential and complementary therapeutic properties of this newly characterised dihydrochalcone, in preventing (i) hypertension and (ii) AGE-linked complications. Endothelial dysfunction, hypertension and atherosclerosis risks are not only associated with a high NADPH activity and superoxide anion overproduction in human blood vessels but also with increased AGEs (Goldin et al., 2006; Guzik et al., 2000). Thus, it will be of interest to assay the capacity of sieboldin to prevent *in vivo* hypertension and to limit atherosclerosis risks. In addition, phloridzin has been widely demonstrated to induce glycosuria and to contribute to overall diminution of glycaemia in animal models (Ehrenkranz et al., 2005). Coupled with the glucosuria-promoting effects of phloridzin, a treatment with sieboldin may be promising in limiting complications associated with diabetes. The two additional tests performed validate the high antioxidant potential of this dihydrochalcone and in addition, provides promising results concerning its potential use as a therapeutic or dietary supplement for oxidative stress related diseases. Hence, sieboldin may be qualified as a “multipotent antioxidant” (Zhang et al., 2006). To the best of our knowledge, this is the first report on the characterisation of sieboldin antioxidant capacities and strong biological activity.

4. Experimental

4.1. General experimental procedures

Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), DPPH (2,2-diphenyl-1-picryl-hydrazyl), fluorescein disodium salt, AAPH (2,2-azobis(2-amidinopropane) dihydrochloride), paraquat dichloride, phloretin, L-ascorbic acid, reduced glutathione, aminoguanidine, D-ribose, bovine serum albumin, dimethyl sulfoxide (DMSO), silica, MeOH and CH₂Cl₂ of HPLC grade were purchased from Sigma–Aldrich (Steinheim, Germany). Apocynin (acetovanillone) was purchased from VWR (Fontenay-sous-Bois, France). HPLC grade-quality acetonitrile and acetic acid were purchased from Carlo Erba (Val de Reuil, France). Phloridzin was obtained from Extrasynthèse (Genay, France). Highly purified and deionised water was obtained from a Milli-Q water system (Millipore, Bedford, USA). UV–visible data were monitored with a microplatespectrophotometer (μ Quant, Bio-Tek, Colmar, France) and fluorescence was read with a FLUOstar Optima (BMG Labtech, Offenbourg, Germany). Analytical HPLC was performed on an HPLC system including a SCM1000 vacuum membrane degasser (ThermoQuest, San Jose, CA, USA), a Surveyor auto-sampler (ThermoFinnigan, San Jose, CA, USA) and a 1100 Series binary pump (Agilent Technologies, Palo Alto, CA, USA). The separated compounds were detected both by a photodiode array detector (1100 Variable Wavelength Detector, Agilent Technologies) and after electrospray ionisation through ion trap mass spectrometry (LCQ Deca, ThermoFinnigan, San Jose, CA, USA) as described by Bernillon et al. (2004). Flash chromatography was performed on a IntelliFlash 310 (Analogix, Burlington, USA). NMR spectra were recorded on an Avance DRX 500 MHz spectrometer (Bruker, Wissembourg, France). Structure of purified compounds prepared in deuteromethanol (CD₃OD) was determined with 1D and 2D ¹H (at 500 MHz) and ¹³C NMR (at 125 MHz) data together with extensive HMBC and HMQC correlation analyses.

4.2. Plant material

Two *Malus domestica* (Baumg.) genotypes were chosen for their contrasting susceptibility to fire blight, a bacterial disease of pome fruits caused by *Erwinia amylovora*, the resistant ornamental Everest and the susceptible rootstock MM106 (Venisse et al., 2002). Experiments were performed on young scions of each genotype grafted on MM106 and grown for at least 6 weeks under green-

house conditions (natural photoperiod and light, temperatures between 17 and 22 °C, 70% relative humidity) before use. Leaf material was obtained from young leaves (F2, second fully expanded leaf, bright green) and mature leaves (F10, the 10th dark-green colored leaf), both harvested on the same growing shoot.

4.3. Analytical HPLC and mass spectrometry

Separation of polyphenols from methanolic extracts was conducted as described previously (Bernillon et al., 2004). The same methanolic extracts used for the DPPH assay were filtered through a 0.45 µm PTFE membrane (Chromafil, Macherey-Nagel, Hoerd, France) before injection. Briefly, samples were injected on a Zorbax Eclipse XDB-C18 column (2.1 mm × 150 mm, 3.5 mm, Agilent Technologies) at 30 °C. The following gradient with a two-solvent system, 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), was applied at a flow rate of 0.2 ml/min: initial, 3% B; 0–5 min, 9% B linear; 5–15 min, 16% B linear; 15–45 min, 50% B linear, followed by washing and reconditioning the column. Quantification of peak areas at 280 nm was achieved by determination of a response coefficient (µmol/OD s) using pure compounds obtained by HPLC purification at the semi-preparative scale as described below.

4.4. Purification of dihydrochalcones from apple leaves

Lyophilized leaves were ground and weighed (14 g), then extracted twice for 24 h with 2 l of acidic methanol (0.1% of acetic acid). Between each step, extracts were vacuum-filtrated on a sintered glass funnel (porosity 4). Filtrates were subjected to evaporation and the resulting powder was mixed with 20 g of silica. Soluble dihydrochalcones were eluted from the silica using CH₂Cl₂/MeOH (5:1). Presence of major dihydrochalcones was checked by analytical LC–MS as described in Section 4.3. Each compound of interest was separated using normal-phase flash chromatography technology (SuperFlash SF 25–40 g CV 52 ml cartridge, Analogix, Burlington, USA) with the following gradient consisting of CH₂Cl₂ (A) and MeOH (B) at a flow rate of 40 ml/min: initial, 5% B; 0–3 min, 10% B; 3–6 min isocratic 10% B; 6–11 min 15% B; 11–20 min isocratic 15% B; 20–25 min 20% B. Polyphenols were detected by UV–visible spectrophotometry. Each fraction was checked by analytical HPLC as described in Section 4.3 before being pooled.

4.5. DPPH scavenging activity

Free radical scavenging activity was assayed as described in Brand-Williams et al. (1995) and Sharma and Baht (2009) with some modifications. Fresh leaf tissue (150 mg) was placed in 2-ml centrifuge tubes with 3 mm-tungsten carbide beads (Qiagen, Courtaboeuf, France), frozen in liquid nitrogen and ground with a mixer mill (MM301, Restch, Haan, Germany) at 30 Hz for 30 s. The resulting powder was then extracted with 1 ml of acidic methanol (0.1% acetic acid) for 15 min in the mixer mill (10 Hz) at room temperature. Homogenates were centrifuged 5 min at 13,000g and the supernatant was transferred to a new 1.5 ml centrifuge tube (called methanol extract afterwards). Pure compound solutions were prepared in methanol at 10× concentrations ranging from 6.25 µM to 3 mM. Assays were performed in microplates (Nunc-Immuno Modules, Nunc, Roskilde, Denmark). Thirty microliter of methanol extract or 10× solutions of each compound was added to 240 µl of methanol in microplate wells. The reaction was started with the addition of 30 µl of a 3.2 mM methanol solution of DPPH and the OD was monitored at 515 nm every minute for 120 min with a UV–visible microplate spectrophotometer. A Trolox standard dilution curve was included as an internal control in each run. Antioxidant activity was evaluated by calculating the

concentration of the tested molecule or raw extract corresponding to 50% reduction of the DPPH radical at steady state (EC₅₀). Trolox equivalent antiradical capacity was calculated as the ratio between the Trolox EC₅₀ and that of the compound/extract tested. Reduction kinetics were also analysed to classify the compounds as slow or rapid (Sanchez-Moreno et al., 1998). The experiment was repeated three times with internal duplicate-measurements for both extracts and compounds ($n = 3$).

4.6. Oxygen radical absorbance capacity

ORAC based on AAPH-initiated chain-oxidation of fluorescein was performed as described in Ou et al. (2001) with minor modifications. Fresh leaf tissue were ground as described for the DPPH assay and extracted with a solvent mixture of water/acetone (50/50). Each compound solution and subsequent dilutions were prepared in potassium phosphate buffer (10 mM, pH 7.4). The reaction was performed in a 96-well black opaque plate (Costar, Cambridge, MA) as follows: 150 µl of 10 nM of fluorescein were added in each well and 25 µl of Trolox or extract or pure compound were pipetted in triplicate. Trolox dilutions ranged from 62.5 to 1000 µM with a 2-fold dilution factor, pure compounds from 12.5 to 100 µM and extracts were diluted 800 and 400 times. The background signal was determined after a 30 min incubation at 37 °C and the reaction was started with the automatic injection of 100 µl of 240 mM AAPH. Fluorescence was measured every 90 s for 10 cycles. Fluorescence data were plotted and the area under the curve (AUC) was calculated as described in Ou et al. (2001). A Trolox equivalent (µM) was calculated with a Trolox regression curve ($Y = 168.18X - 20.269$, $R^2 = 0.9977$ with $X = \text{AUC sample} - \text{AUC blank}$). Each measurement was performed in triplicate. The analysis was carried out on three independent leaf extracts ($n = 3$) and two different dilutions of the purified compound with two independent measurements ($n = 4$).

4.7. Tolerance to herbicide-derived photooxidative stress

The tolerance to a photooxidative herbicide (paraquat) was assessed by monitoring electrolyte leakage by conductimetry as described previously (Brisset and Paulin, 1991) with the following modifications. Ten leaf discs (0.7 cm in diameter, 40–50 mg of total fresh weight) from young F2 leaves of Evereste and MM106 were vacuum-infiltrated with 1, 10 or 100 µM paraquat (methyl viologen; Donahue et al., 1997) prepared in 1 mM MES buffer after surface-sterilisation (in 1.25% active Cl). The controls consisted of 1 mM MES infiltrated leaf discs. The discs were then blotted dry and immediately transferred for incubation in fresh 1 mM MES buffer with continuous stirring at 25 °C under artificial light provided with a GW84011M Mercurio floodlight (170 µmol/m²/s, Gewiss, Les Ulis, France). Conductivity of the bathing MES solution was measured every 2 h for 24 h. The experiment was repeated four times, each measurement being performed in triplicate within each experiment.

4.8. Vasorelaxant effects of dihydrochalcones

Adult male Wistar rats (Iffa-Credo, L'Arbresle, France) were anesthetized (pentobarbital sodium, 50 mg/kg ip). Segments of first order mesenteric arteries (2 mm long) were dissected and mounted on a wire myograph (DMT, Aarhus, Denmark) in physiological salt solution as described previously (Loufrani et al., 2008). Tensions were monitored using the v3.7.2 of the AcqKnowledge software (BIOPAC Systems, Inc., Goleta, CA). A control of endothelium integrity was achieved by testing acetylcholine-induced relaxation as described in Loufrani et al. (2008). A cumulative concentration–response curve to phenylephrine (10^{-9} – 10^{-4} M) was performed. After

washout, dihydrochalcones prepared in DMSO and diluted in ultra-pure water were added to the vessel at a final concentration of 10^{-5} M. Sieboldin and phloridzin inhibitory activities were compared to that of control compounds, tempol (10^{-5} M) and apocynin (10^{-5} M), used under the same conditions. Vessels were then allowed to stabilize for 30 min and a new cumulative concentration–response curve to phenylephrine was performed. Each experiment was repeated three times, corresponding to three different animals. Cumulative concentration–response curves to sieboldin or phloridzin (10^{-9} – 10^{-5} M) after precontraction of the artery with 10^{-6} M phenylephrine were also performed.

4.9. Inhibition of advanced glycation end products (AGEs) formation

A Maillard fluorescence-based assay adapted from Vinson and Howard (1996) was optimized to screen compounds able to inhibit the formation of AGEs. The assay involved incubating bovine serum albumin (BSA) (10 mg/ml) with D-ribose (0.5 M) and the tested compound (10^{-5} – 10^{-2} M) in a Na-phosphate buffer (50 mM pH 7.4). Solutions were incubated in black microtitre plates (96 wells) at 37 °C for 24 h in a closed system. AGEs fluorescence (excitation: 370 nm; emission: 440 nm) was measured using a spectrofluorimeter Infinite M200 (Tecan, Lyon, France). To avoid quenching phenomena, the fluorescence resulting from incubation in the same conditions with BSA (10 mg/ml) and the tested compound or extract was subtracted from each measure. Tests were performed in triplicate. The negative control, i.e. 100% inhibition of AGEs formation consisted of wells with only BSA. The positive control, i.e. no inhibition of AGEs formation consisted of wells with BSA (10 mg/ml) and D-ribose (0.5 M). The final volume assay was 100 µl. The compound concentration for 50 percent inhibition (IC_{50}) was calculated from the data and compared with that of the reference compound aminoguanidine (Reddy and Beyaz, 2006).

4.10. Statistical analysis

A Bartlett's test was performed using R (R Development Core Team, 2008) to test for heteroscedasticity. When heterogeneity of variances was detected ($p < 0.05$ for the Bartlett's test), data were represented with box-and-whisker plots (Chambers et al., 1983). For each plot, the two hinges correspond to the first and the third quartile, median and mean are symbolised by a horizontal line and a cross, respectively. Such a representation has the advantage of giving information about biological variability. The whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box. Notches represent a 95% confidence interval for the medians. Thus, if the notches of two plots do not overlap, medians may be considered significantly different, allowing to conclude about statistical significance like in classical parametric tests used for homoscedastic data. Data for which homogeneity of variances was verified ($p > 0.05$ for the Bartlett's test) were analysed using a one-way anova and Student's *t* pairwise comparison of Benjamini-Hochberg adjusted *p*-values of means.

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