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COMPARATIVE STUDY OF RADICAL SCAVENGER AND ANTIOXIDANT PROPERTIES OF PHENOLIC COMPOUNDS FROM *VITIS VINIFERA* CELL CULTURES USING *IN VITRO* TESTS

Bernard FAUCONNEAU*, Pierre WAFFO-TEGUO†, François HUGUET*, Laurence BARRIER*, Alain DECENDIT†, Jean-Michel MERILLON†

*Center for Study and Research on Xenobiotics, EA 1223, Faculty of Pharmacy, Poitiers, France.
†Study Group for Natural Substances of Therapeutic Interest, EA 491, Faculty of Pharmacy, Bordeaux, France.

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Summary

Vitis vinifera cell suspensions were used to isolate and characterize the flavonoids (anthocyanins, catechins) and non-flavonoids (stilbenes) found in red wine. Furthermore, we showed that astringin is produced although this stilbene has not previously been reported to be a constituent of *V. vinifera* or wine. The ability of these compounds to act as radical scavengers was investigated using 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical. Antioxidant activities were assessed by their capacity to prevent Fe^{2+}-induced lipid peroxidation in microsomes and their action on Cu^{2+}-induced lipid peroxidation in low-density lipoproteins. The results showed that astringin has an important antioxidant effect similar to that of *trans*-resveratrol, and a higher radical scavenger activity than the latter. Astringinin appeared to be more active. These data indicate that phenolic compounds (stilbenes, catechins, anthocyanins) exhibit interesting properties which may account in part for the so-called "French paradox," i.e. that moderate drinking of red wine over a long period of time can protect against coronary heart disease.

Key Words: **antioxidants, phenolic compounds, wine, free radicals**

In most countries, though not in some regions of France, a high intake of saturated fats is strongly correlated with high mortality from coronary heart disease (CHD). The Southern French have a very low incidence of CHD despite having a high fat diet and smoking habits. This so-called "French paradox" has been attributed in part to wine consumption (1). It has frequently been suggested that the constituents of red wine, particularly polyphenolic substances, help protect against CHD. In fact, there is increasing evidence that oxidized low-density lipoproteins (LDL) may be responsible for promoting atherogenesis. In this context, Frankel *et al.* (2, 3) have shown that total phenolic compounds extracted from red wine inhibit the oxidation of human LDL *in vitro*, which could account for the French paradox. In addition, red wine consumption results in a reduced propensity of human plasma and LDL to undergo lipid peroxidation (4).

Flavonoids constitute the main polyphenolic compounds in red wine and there are two major classes: anthocyanins (0.2-0.8 g/l) and flavanols, including catechin monomers and oligomers (1-3 g/l) (5). In addition, wine contains non-flavonoids, particularly stilbenes (typically 0.02-0.04 g/l) (6). The latter have been the subject of much study since grapes and red wine are

Corresponding author: Bernard Fauconneau, Center for Study and Research on Xenobiotics, Faculty of Pharmacy, F-86005 Poitiers, France. FAX: 33.5.49.44.38.34

probably the most important foodstuffs containing these substances (7, 8). Moreover, traditional Chinese medicine uses a preparation from the roots of *Polygonum cuspidatum*, which contains piceid, to treat atherosclerosis (9).

We first isolated and characterized flavonoids (catechins and anthocyanins) and stilbenes (*cis*- and *trans*-resveratrol and their glycosides) from *V. vinifera* cells (10-12) and also showed that astringin is produced although this stilbene has not previously been reported to be a constituent of *V. vinifera* or wines (12). Three tests were used to assess the properties of these compounds: their capacity to prevent Fe^{2+}-induced lipid peroxidation in microsomes (a membrane preparation rich in polyunsaturated fatty acids), their action on Cu^{2+}-induced lipid peroxidation in LDL, and their direct scavenging effect on a stable free radical, 1,1-diphenyl-2-picryl-hydrazyl (DPPH).

Test on LDL was the most relevant one since high levels of LDL are associated with increased atherosclerosis. Factors which reduce LDL concentrations, such as a lower intake of dietary saturated fatty acids, can reduce atherogenesis. There is increasing evidence that LDL oxidation greatly increases the capacity of macrophages, endothelial cells and monocytes to bind and digest LDL via specific receptors. This accelerates foam cell development and the accretion of plaque material, i.e. atherogenesis. Hence, it is conceivable that oxidative stress accelerates atherogenesis by enhancing LDL oxidation and increasing its accumulation in foam cells. The presence of a chain-breaking antioxidant can interfere with this peroxidation process by reducing the alkoxyl or peroxyl radical to alkoxides or hydroperoxides, respectively, so that the hydroperoxides re-enter the cycle until the antioxidant is consumed (13).

Methods

Cell cultures

Cell cultures of *Vitis vinifera* (L.) cv. Gamay Fréaux var. Teinturier were established in 1978 from pulp fragments of young fruits and provided by C. Ambid (ENSA, Toulouse, France).

Chemicals

Cis- and *trans*-piceid, *trans*-resveratrol, astringin, catechin, epicatechin, peonidin 3-O-β-glucoside and malvidin 3-O-β-glucoside were isolated from *V. vinifera* cell suspension cultures and characterized using spectrometric methods (11, 12). *Cis*-resveratrol and astringinin were obtained by enzymatic hydrolysis of cis-piceid and astringin, respectively. All these compounds were dissolved in HPLC grade methanol and added to the reaction mixture to be tested.

Antioxidant activity

- Tests on microsomes

Rat liver microsomes were prepared by tissue homogenization with 5 volumes of ice-cold 0.15 M KCl. Microsomal fractions were isolated in Tris-HCl 0.05 M / KCl 0.15 M, pH 7.4, by removal of the nuclear fraction at 900 g for 15 min, removal of the mitochondrial fraction at 15,000 g for 15 min and sedimentation at 125,000 g for 30 min. Fractions were washed twice in the buffer by centrifugation, with subsequent sedimentation at 125,000 g for 15 min. Microsome pellets were stored at -80° C for a maximum of one month.

The inhibitory effect on lipid peroxidation was assessed by measuring thiobarbituric reactive substances (TBARS), mainly constituted by malonaledialdehyde (MDA). For tests, microsomal fractions were diluted with 0.05 M Tris-HCl, pH 7.4, containing KCl (0.15 M). The final protein concentration in the incubation mixture was determined by the Bradford method (14) using Coomassie blue and amounted to 0.75 mg/mL. The mixture was then preincubated in a shaking water-bath at 37° C for 10 min with different concentrations of each one of the compounds studied. Lipid peroxidation was initiated with 10 μM $FeCl_2$. After incubating the samples for 30 min, 1 mL 20% trichloroacetic acid (TCA) was added to stop the lipid peroxidation reaction. After centrifugation, 1 mL 0.67 % thiobarbituric acid (TBA) was added in the supernatant followed by 15 min incubation in boiling water. The absorbance was measured at

532 nm for TBARS determination (15). Measurements were performed at least in triplicate. Inhibition of lipid peroxidation was expressed as a percentage, and the effective concentration 50% (EC_{50}) was obtained from the inhibition curve.

- Tests on low density lipoproteins (LDL)
Blood (20 mL for each donor) was collected by venipuncture in lithium heparinate from 3 consenting healthy volunteers, pooled and centrifuged at 1,500 g at +4°C. Plasma LDL were prepared by sequential flotation ultracentrifugation in KBr and dialyzed against a phosphate buffer. They were stored at -80°C for a maximum of 2 weeks.

Lipoproteins were pre-incubated in the presence of different concentrations of each one of the compounds studied at 37°C for 1 h in a shaking water-bath. The final protein concentration in the incubation mixture amounted to 0.1 mg/mL (14). Lipid peroxidation was then initiated with 5 µM $CuSO_4$, and the mixture was incubated for 2 h. Then, 1 mL TCA (20%) was added. After centrifugation, 1 mL 0.67% TBA was added in the supernatant, followed by 15 min incubation in a boiling water bath. TBARS was measured at 532 nm using the colorimetric methods (15). Measurements were performed at least in triplicate, and inhibition of coloration was expressed as a percentage. The EC_{50} was obtained from the inhibition curve.

We verified that there was no interference due to anthocyanin coloration by using the high-performance liquid chromatography (HPLC) method of Chirico (16), with slight modifications. In short, after defecation of the samples by perchloric acid, the supernatant was neutralized by kalium carbonate 5 M. Samples were centrifuged for 15 min at 2,000 g, and 250 µL of supernatant were added to 750 µL phosphoric acid 0.44 M. After a 10-min pause, derivatization was performed with 250 µL thiobarbituric acid 0.67 % (w/v). The samples were incubated for 45 min at 100°C. Twenty microliters were then injected onto a column (Lichrospher RP18 Merck; 250 x 4 mm, 5 µm) using a Merck L 6200 pump. The samples were eluted with a mobile phase consisting of 65% 0.05 M kaliumdihydrogenophosphate, 15% methanol and 20% acetonitrile at a flow rate of 0.9 mL/min. A sharp peak corresponding to a $(TBA)_2$-MDA adduct eluted at a retention time of 10 min was detected by a visible detector set at 532 nm (Shimadzu RF-551). A tetramethoxypropane standard was used to quantify MDA levels.

Radical scavenging effect
The free radical scavenging capacity of the phenolic compounds was determined using DPPH (17). An ethanol DPPH solution (100 µM) was mixed with different concentrations of each one of the compounds studied, and the absorbance change at 515 nm was measured 10 min later with a spectrophotometer (Uvikon 940, Kontron). Measurements were performed at least in triplicate. Inhibition of coloration was expressed as a percentage, and EC_{50} was obtained from the inhibition curve.

Statistical analysis
Analysis was performed using ANOVA completed by Student's *t*-test. A value of $p<0.05$ was considered significant.

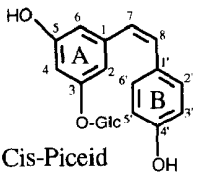
Results and Discussion

In this study, the tests used allowed two properties of these compounds to be assessed (18, 19): direct antioxidant activity, as regards induction of lipid peroxidation on microsome and LDL tests, and the direct scavenging effect on a stable free radical, DPPH.

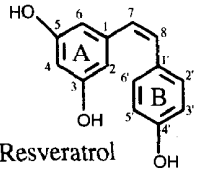
To be considered as an antioxidant, a polyphenol must satisfy two basic conditions: first, when present at a low concentration relative to the substrate to be oxidized, it can delay or prevent autooxidation or free radical-mediated oxidation; secondly, the resulting free radical formed after scavenging must be stable upon further oxidation (20).
Fig. 2 gives an example of curves obtained with astringin on the different tests in one experiment. The mean ± SD obtained in at least three experiments are given in table I.

NON-FLAVONOIDS

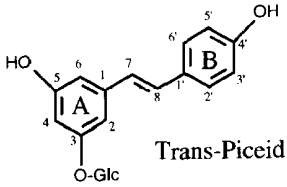
STILBENES



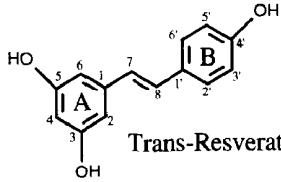
Cis-Piceid



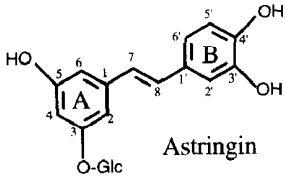
Cis-Resveratrol



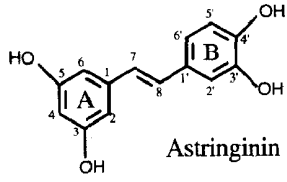
Trans-Piceid



Trans-Resveratrol



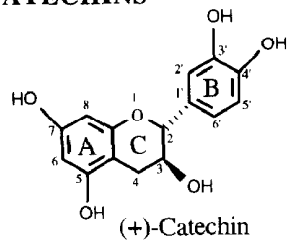
Astringin



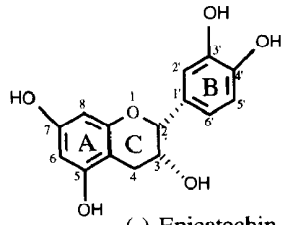
Astringinin

FLAVONOIDS

CATECHINS

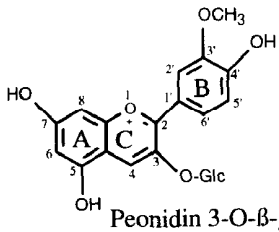


(+)-Catechin

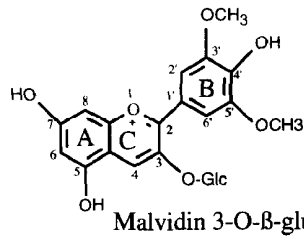


(-)-Epicatechin

ANTHOCYANINS



Peonidin 3-O-ß-glucoside



Malvidin 3-O-ß-glucoside

Fig. 1

Structures of different Phenolic Compounds extracted from *V. vinifera* Cell Cultures.

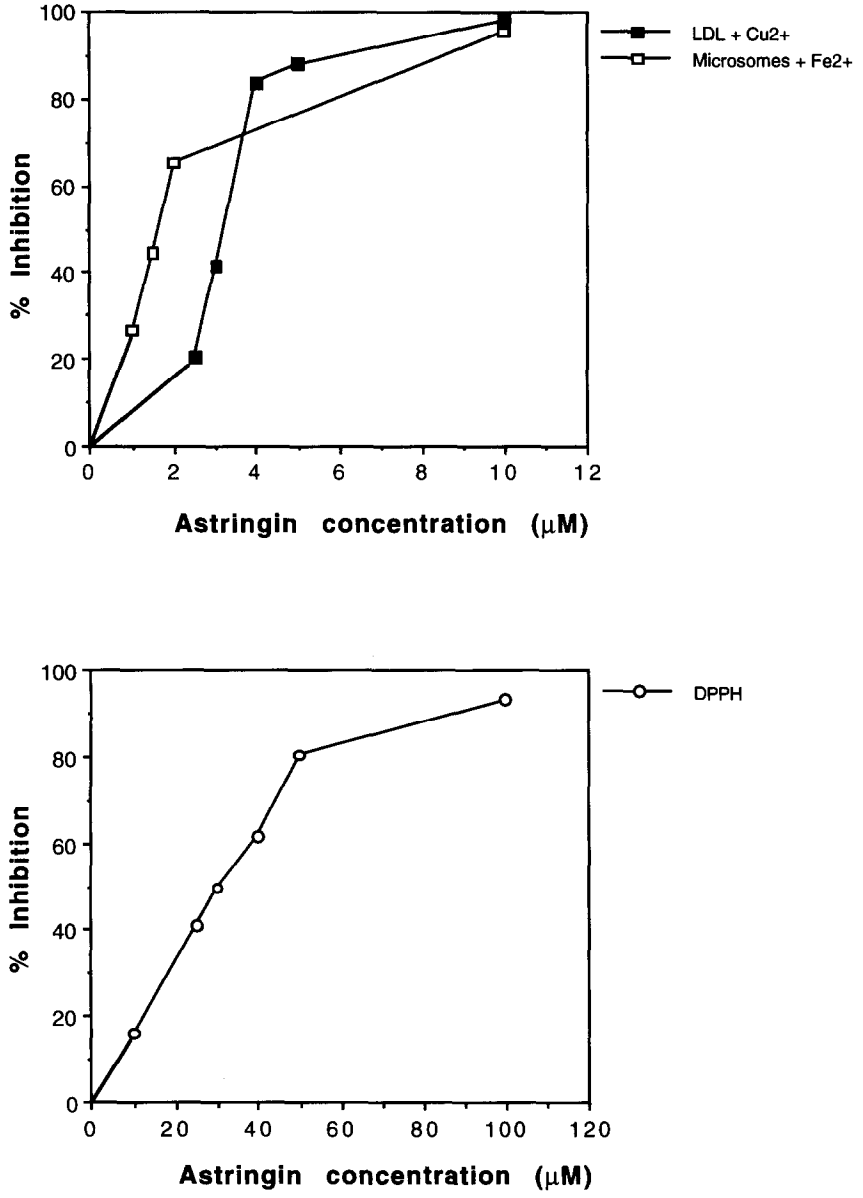


Fig. 2

Activity of astringin in one experiment on LDL, microsome, and DPPH tests. EC_{50} is determined from the cuve.

TABLE I

Antioxidant Activities of Phenolic Compounds extracted from *Vitis vinifera* Cell Cultures

	Microsomes EC_{50} (μM)	**LDL** EC_{50} (μM)	**DPPH** EC_{50} (μM)
NON-FLAVONOIDS			
Stilbenes			
Cis-piceid	16.0 ± 0.5	16.6 ± 1.5	140 ± 12
Trans-piceid	21.3 ± 2.7	19.3 ± 3.2	200 ± 19
Cis-resveratrol	18.1 ± 0.3	19.0 ± 2.0	97.0 ± 4.2
Trans-resveratrol	3.0 ± 0.2	2.6 ± 0.3	74.0 ± 5.3
Astringin	1.9 ± 0.4	3.1 ± 0.2	30.6 ± 2.1
Astringinin	1.0 ± 0.1	1.9 ± 0.2	29.0 ± 1.4
FLAVONOIDS			
Catechins			
(+)-Catechin	2.0 ± 0.3	1.9 ± 0.1	20.2 ± 1.6
(-)-Epicatechin	1.1 ± 0.2	1.0 ± 0.1	15.7 ± 1.5
Anthocyanins			
Peonidin-3-O-ß-glucoside	3.4 ± 0.6	3.2 ± 0.7	49.1 ± 5.2
Malvidin-3-O-ß-glucoside	3.7 ± 0.7	3.8 ± 0.8	42.1 ± 4.4
DRUG REFERENCE			
Trolox	5.0 ± 0.3	4.7 ± 0.4	10.1 ± 0.5

Each value is the mean of at least three independent experiments ± SD.

Tests on microsomes

The results observed in this system were absolutely identical to those found in the LDL/Cu^{2+} system. Although inhibition of iron-induced microsomal lipid peroxidation is a valuable test for antioxidant capacity in membranes, it cannot be excluded that a polyphenolic-iron complex develops and is incapable of forming free radicals.

- Tests on LDL

In this test, the activity of *cis*-piceid was similar to those of *trans*-piceid and of the aglycone of *cis*-piceid (i.e. *cis*-resveratrol). Conversely, the activity of the aglycone of *trans*-piceid (i.e. *trans*-resveratrol) was seven times greater than that of *trans*-piceid ($p<0.001$). When a hydroxyl group was added on the B ring (i.e. astringin), the activity became 6 times greater than that of *trans*-piceid ($p<0.001$). Astringinin, which possesses a supplementary OH as compared to *trans*-resveratrol, was more efficient than *trans*-resveratrol ($p<0.05$), indicating the major importance

of the OH group, as previously noted (20). Furthermore, astringinin was more efficient than Trolox, the water soluble vitamin E analog ($p<0.01$). As a general rule, glycosylation in position 3 on ring A decreases the activity of aglycone molecules. However, as the human digestive tract is known to have glycosidase activity (19), glycosides could release aglycones on ingestion and increase the antioxidant effect *in vivo*.

Epicatechin was twice as active as catechin ($p<0.01$), and no differences were observed between the two anthocyanins tested.

- *Tests on DPPH*

Cis-piceid was more efficient than *trans*-piceid ($p<0.05$), but considering the activities of the aglycones of these compounds, *trans*-resveratrol was more potent than *cis*-resveratrol ($p<0.05$). Radical scavenger activity was increased with the aglycone forms of these products (*cis*-resveratrol vs *cis*-piceid and *trans*-resveratrol vs *trans*-piceid; $p<0.05$ and $p<0.01$, respectively), but the activity of astringinin was close to that of astringin. When an OH group was added on ring B (i.e. astringin = OH *trans*-piceid; astringinin = OH *trans*-resveratrol), the effect was increased ($p<0.005$). Hence, the scavenger properties of these compounds are associated with their ability to form stable radicals, and it is well-known that aromatic compounds containing hydroxyl groups, especially those with an ortho-dihydroxy function on the B ring, appear to be important scavengers, as reported for flavonoids (21).

Catechins showed the greatest radical-scavenging action, while the two anthocyanins were only half as potent.

The DPPH test provided information about the reactivity of the tested compound with a stable free radical. Because of its odd electron, the DPPH radical showed a strong absorption band at 515 nm in visible spectroscopy (a deep purple color). As this electron is paired off in the presence of a free radical scavenger, absorption vanishes and the resulting decoloration is stoichiometric with respect to the number of electrons taken up. This bleaching of DPPH absorption, which occurs when the odd electron of the radical is paired, is thus representative of the capacity of the compounds to scavenge free radicals independently of any enzymatic activity.

Taken together, these results show that the chemical criteria essential for the antioxidant activities of these polyphenolic compounds (flavonoids and stilbenes) are (i) the presence of the catechol structure or 4'-hydroxy in ring B and (ii) the presence of the *meta*-hydroxy structure in ring A. For flavonoids, glycosylation of the hydroxyl group at C-3 does not seem to change antioxidant activity notably, as reported by Teisseidre *et al* (22).

This study demonstrated the trapping effect and antioxidative properties of astringin, a phenolic compound newly extracted from *V. vinifera*, on LDL oxidized by Cu^{2+}, a relevant model. These data support previous work (3) suggesting that various phenolic substances in red wine may provide a protective effect against atherogenesis through their antioxidative properties over a long period of moderate intake of red wine (2 to 3 glasses per day). This consumption corresponds to about 300 to 1,200 mg of red wine polyphenols, which is much greater than the recommended intake of antioxidant vitamins. To assess the protective effect of wine phenolics more thoroughly, we have recently undertaken the production of ^{13}C labelled phenolic compounds found in red wine in order to investigate their absorption, *in vivo* metabolism and pharmacokinetics in human.

Acknowledgments

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