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Activity and Mechanism of the Antioxidant Properties of Cyanidin-3-O-β-glucopyranoside

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In the present study, the antioxidant activity, the interaction with reactive oxygen species and the redox potential of cyanidin-3-*O*-β-glucopyranoside (C-3-G), the main anthocyanin present in juice of pigmented oranges, were evaluated in detail. C-3-G effects on low density lipoproteins (LDL) oxidation induced by 40 μM Cu^{2+} at a pH of 7.4 were compared with those of resveratrol and ascorbic acid, two other natural antioxidants. All cyanidin-3-*O*-β-glucopyranoside concentrations used (1, 2, 5, 10, 20, 50, 100 and 200 μM) inhibited malondialdehyde (MDA) generation (an index of lipid peroxidation), the inhibition being significantly higher than that obtained with equal concentrations of resveratrol and ascorbic acid (IC_{50}=6.5 μM for C-3-G, 34 μM for resveratrol and 212 μM for ascorbic acid). Experiments of LDL oxidation performed at a pH of 5.0 or 6.0 showed that C-3-G antioxidant activity is not influenced by pH variations between 5.0 and 7.4. This suggests that metal chelation, exerted by C-3-G through the eventual dissociation of its phenolic groups, plays a minor role in its protective mechanism. The presence of C-3-G produced significantly higher protective effects of pigmented orange juice (obtained from Moro cultivar) with respect to blond orange juice, when tested on copper-induced LDL oxidation. The evaluation of the direct interaction with reactive oxygen species (H_2O_2, $^{-}O_2$, $OH^{\cdot}$), demonstrated that C-3-G is quickly oxidized by these compounds and it is, therefore, a highly efficient oxygen free radical scavenger. The powerful C-3-G antioxidant activity is in excellent agreement with the very negative redox potential (−405 mV), determined through direct current cyclic voltammetry measurements.

On the basis of these results, C-3-G should be considered as one of the most effective antioxidants that can be assumed with dietary plants; therefore, pigmented oranges represent a very relevant C-3-G

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source because of the high content of this anthocyanin in their juice.

Keywords: Cyanidin-3-*O*-β-glucopyranoside; Anthocyanins; Reactive oxygen species; Nutritional antioxidants; Pigmented oranges

Abbreviations: C-3-G, cyanidin-3-*O*-β-glucopyranoside; ROS, reactive oxygen species; LDL, low-density lipoproteins; MDA, malondialdehyde

INTRODUCTION

Cyanidin-3-O-*β*-glucopyranoside (C-3-G) is a natural compound distributed in several fruits and vegetables, such as strawberry, blackberry, rhubarb, cherry, red cabbage, red onion, cranberry, etc.,[1,2] as well as in pigmented oranges (named Moro, Sanguinello and Tarocco),[3] typically growing in Sicily and also in Malta. It is classified as an anthocyanin, which is the largest class of water-soluble compounds in plants, where they are responsible for the brilliant colors (red, orange, blue) of flowers and fruits. In plant kingdom, the biological role of anthocyanins is to participate to attract animals in pollination and seed dispersal. The chemical structure of anthocyanins (including C-3-G) is constituted by the basic tricyclic flavylium cation, on the B ring of which various chemical groups are located. Due to the simultaneous presence of several OH groups, anthocyanins are, therefore, polyphenols. Glycosylation occurs on specific carbon atoms (3, 5, 7, 3′ and 5′) of the rings and greatly contributes to increase the stability of the anthocyanin molecule. C-3-G has 3′-and 4′-OH groups on the B ring (for a total of four hydroxyl groups) and a β-D-glucose residue linked by a 3-*O*-β glycosyl bond with the C ring (Fig. 1). Anthocyanins are particularly reactive because of their natural electron deficiency which also render these compounds very sensitive to pH and temperature changes.[4]

In the last years, great attention was given to the possible protection exerted by natural antioxidants present in dietary plants towards tissue injury mediated by reactive oxygen species

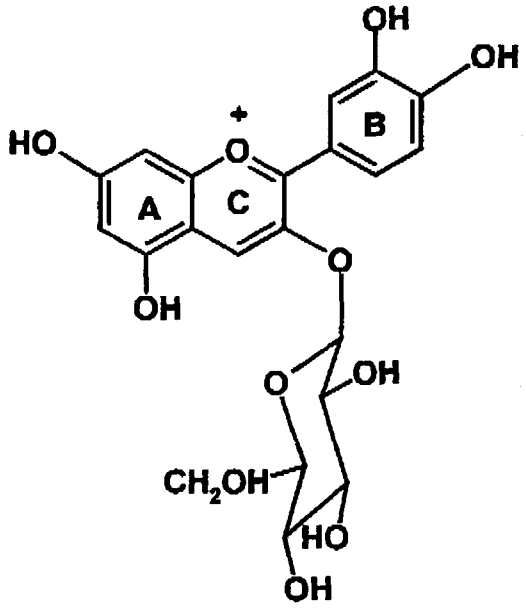

FIGURE 1 The chemical structure of cyanidin-3-*O*-β-glucopyranoside.

(ROS),[5,6] with particular focus on the effects of flavonoids and polyphenols.[7–9] Hence, even if some concerns existed because of their instability at physiological pH, anthocyanins were included in the list of natural compounds known to work as powerful antioxidants,[4,10] the potency of which depends on their different chemical structure. In fact, it was reported that, by varying positions and types of chemical groups on the aromatic rings of anthocyanins, it is possible to modulate the capacity to accept unpaired electrons from radical molecules, and thus to increase or decrease the corresponding antioxidant activity.[4,10] Notwithstanding, variable results did not allow to find a definitive structure-function correlation for anthocyanins.[1,11] Among anthocyanins, C-3-G was indicated as one of the most effective antioxidants[4,10,12–15] and its mechanism of action, at least in metal-dependent ROS generating systems, was in great part ascribed to its capacity of complexing divalent metal ions necessary for generating ROS through the Fenton reaction.[4]

However, in spite of the relative abundance of data, some basic information fundamental for a better characterization of the antioxidant profile of C-3-G, such as the value of its redox potential, have not yet been determined.

In this study, we have reported data concerning the dose-response effect of C-3-G to inhibit oxidation of human LDL induced by Cu^{2+} (in comparison with other known antioxidants such as resveratrol and ascorbic acid) and we demonstrated that C-3-G acts as a real antioxidant, and not as a simple metal-chelating compound, because of its peculiar redox potential. In addition, we described experiments in which, due to the presence of C-3-G, pigmented orange juice showed higher antioxidant capacity than blond orange juice.

MATERIALS AND METHODS

Chemicals

Purified human LDL, resveratrol and ascorbic acid were obtained from Sigma (St. Louis, MO, USA). C-3-G was purchased from Polyphenols AS Laboratories (Hanabryggene Technology Centre, Sandnes, Norway). Tetrabutylammonium hydroxide, used as the ion-pairing reagent for the HPLC detection of MDA and ascorbic acid, was obtained as a 55% water solution from Nova Chimica (Cinisello Balsamo, Milan, Italy). All other reagents were of the highest purity available from commercial sources.

Oxidation of Human LDL

Lyophilized LDL were resuspended in water (2 mg protein/ml) and extensively dialyzed against 20 mM $CH_3COONH_4^+$ buffer, pH 7.4, to remove any phosphate and EDTA traces. LDL suspension was used at a final concentration of 1 mg/ml. Oxidation was started by the addition of Cu^{2+} (40 μM final concentration) to the protein suspension and prosecuted by incubation at 37°C for 24 h. The inhibitory effects on lipid peroxidation of C-3-G, resveratrol and ascorbic acid were evaluated by either incubating 40 μM Cu^{2+}-challenged LDL suspensions with increasing concentrations of antioxidants (1, 2, 5, 10, 20, 50, 100 and 200 μM) and for a fixed time (24 h), or by following the time-course of 40 μM Cu^{2+}-challenged LDL suspensions incubated with the most effective dose of any of the aforementioned antioxidants (200 μM). In this latter case, withdrawals of the different LDL suspensions were effected immediately before (0 time), and 0.5, 4, 8 and 24 h after Cu^{2+} supplementation. In addition, experiments for evaluating the influence of pH on antioxidant efficacy of C-3-G were also performed. For this purpose, LDL were suspended in 20 mM $CH_3COONH_4^+$, pH 5.0 or 6.0 and incubated at 37°C for 24 h with various C-3-G concentrations (1, 2, 5, 10, 20, 50, 100 and 200 μM). For any pH values, LDL incubated in the presence of buffer only deserved as controls.

Since C-3-G represents about 90% of total anthocyanins of pigmented oranges,[16,17] we performed LDL oxidation (induced by incubating the protein suspension with 40 μM Cu^{2+} for 8 h at 37°C) in presence of different volumes of fresh orange (*Citrus sinensis* (L.) Osbeck) juices obtained from pigmented (Moro) or blond (Washington navel) varieties, in order to asses possible different antioxidant power of juices, just based on the presence or absence of C-3-G. The Moro cultivar was chosen because of its highest anthocyanin concentration with respect to the other pigmented orange varieties, as clearly reported by previous observations.[16] Juices were prepared by squeezing oranges with a domestic squeezer; particulate debris was removed by centrifugation at 20,690 g for 10 min at 4°C and subsequent filtration through a 0.45 μm Millipore filters. To allow a successive comparison of results, the antioxidant profiles of pigmented and blond juices were performed by quantifying their total anthocyanin[17] and ascorbic acid content.[18] On the basis of the total anthocyanin concentration,

juices (both pigmented and blond) were appropriately diluted before their addition to LDL suspension, so that the final C-3-G concentrations obtained were similar to those used in experiments in which the pure compound was used.

All LDL incubations were stopped by adding a double volume of HPLC-grade acetonitrile and samples were processed for the HPLC determination of MDA to evaluate the extent of LDL lipid peroxidation.

HPLC Analysis of MDA

After acetonitrile addition, samples of LDL suspensions were extracted twice with chloroform (2:1; v:v) and, after each extraction, they were centrifuged at 20,190 g for 5 min at 4°C. The upper aqueous phase was collected, filtered through a 0.45 μm Millipore filter and then loaded (200 μl) onto a C-18, 250 × 4.6 mm, 5 μm particle size column (Kromasil, Bohus, Sweden) for the HPLC detection of MDA. The HPLC apparatus consisted of a SpectraSystem P2000 pump (ThermoQuest, Rodano, Milan, Italy) connected to a highly sensitive UV6000 LP diode array spectrophotometric detector (ThermoQuest, Rodano, Milan, Italy), equipped with a 5 cm light path flow cell and set up between 200 and 300 nm for data acquisition. Determination of MDA was directly effected on the organic solvent-extracted samples according to an ion-pairing method described in detail elsewhere,[18] which does not require sample derivatization prior to HPLC analysis. MDA quantification was calculated at 267 nm wavelength (MDA maximum of absorbance).

Spectrophotometric Experiments

To assess whether the C-3-G capacity to inhibit copper-induced LDL oxidation were due to an interaction with ROS, C-3-G was challenged with H_2O_2, xanthine+xanthine oxidase, and Fe^{2+} + H_2O_2. In order to choose a wavelength where change of the redox state of C-3-G were clearly observable, 100 μM C-3-G was preliminarily dissolved in oxygen or nitrogen-saturated 20 mM phosphate buffer, pH 7.0, and incubated at 37°C. Absorption spectra were recorded every 15 min from 200 to 700 nm wavelength. In the visible region, two maxima of absorbance at 438 and 548 mn were observed, both undergoing to progressive absorbance decrease but in the oxygen-containing solution only. Since the peak at 438 nm showed a more pronounced absorbance decrease it was selected as the wavelength for evaluating C-3-G oxidation. In the reaction with hydrogen peroxide, 100 μM C-3-G was mixed in a quartz cuvette (1 ml final volume) with 20 mM KH_2PO_4, pH 7.0, and 1, 2, 5, 10, or 20 mM H_2O_2. Absorbance variations were followed at 438 nm and the initial rate of the reaction was considered. To evaluate the interaction with $^{-}O_2$, C-3-G (1, 2, 5, 10, 20, 50, 100 and 200 μM) was mixed in a quartz cuvette (1 ml final volume) with 300 μM xanthine, 0.07 U xanthine oxidase, 100 μM cytochrome c, 20 mM KH_2PO_4, pH 7.0. Generation of $^{-}O_2$ by xanthine oxidase was followed at 550 nm by monitoring the velocity of absorbance increase due to superoxide-dependent cytochrome c reduction, with and without C-3-G. To verify the effect of hydroxyl radicals, 100 μM $FeC1_2$ was reacted with 20 mM KH_2PO_4, pH 7.0, for 10 min at room temperature in a quartz cuvette (1 ml final volume), in order to allow iron chelation by inorganic phosphate. C-3-G (100 μM) was then mixed to the solution and the cuvette placed in the spectrophotometer. Quick addition of variable H_2O_2 concentrations (0.25, 0.5, 1, 2, 5, 10 and 20 mM) to generate $OH^{\cdot}$ through the Fenton reaction, was then performed and the initial rate of C-3-G oxidation was followed at 438 nm.

All spectrophotometric measurements were carried out at 37°C, using a Beckman DU-640 spectrophotometer.

Direct Current Cyclic Voltammetry Measurements

To establish the redox potential of C-3-G, direct current (DC) cyclic voltammetry experiments were performed at 25°C in a glass microcell (1 ml sample volume) equipped with a reference standard calomel electrode (SCE) (Amel, Milan, Italy), a Pt wire as the counter-electrode and a pyrolytic graphite electrode of 3 mm diameter as the working electrode (Amel, Milan, Italy). A 433/W multipolarograph (Amel, Milan, Italy), interfaced with a PC, was employed for voltammetric measurements. Before the voltammetric experiments, the C-3-G solution (1.5 mg/ml in 50 mM phosphate buffer, pH 7.0, containing 50 mM NaCl as supporting electrolyte) was deaerated for 20–30 min by a gentle flow of pure nitrogen, maintained just above the solution surface.

Total Anthocyanin and Ascorbic Acid Determination in Orange Juices

Total anthocyanin content in pigmented orange juices was determined spectrophotometrically, according to the method of Rapisarda *et al.*,[17] while ascorbic acid was determined by injecting 10 µl of clear juices onto the above mentioned HPLC column and separated by using the same chromatographic conditions used for separating MDA.[18] Ascorbic acid was quantified at 265 nm wavelength (ascorbate maximum of absorbance) by comparing peak areas of chromatographic juice runs with those of ultra-pure standard with known concentrations.

Statistical Analysis

Differences amongst the various antioxidants, as well as between pigmented and blond orange juices, were analyzed by the analysis of variance and by the two-tailed Student's *t*-tests for unpaired samples; a p value of less than 0.05 was considered significant.

RESULTS

Effect of C-3-G on LDL Oxidation

In Table I is reported the effect of increasing C-3-G concentrations on the time-course of LDL peroxidation induced by 40 µM Cu^{2+}, evaluated by determining MDA generation as an end product of lipid peroxidation. The results indicate that the inhibitory capacity of C-3-G was independent of the time of incubation and it

TABLE I Dose-response effect of cyanidin-3-*O*-β-glucopyranoside (C-3-G) on the time-course of copper-induced LDL oxidation. Protein suspensions were incubated with 40 µM Cu^{2+} for different times at 37°C in 20 mM ammonium acetate, pH 7.4. Levels of lipid peroxidation were estimated by direct HPLC measurements of MDA. Control LDL was incubated in presence of buffer only. Each value is the mean (SD) of five experiments

C-3-G added (µM)	MDA (µM), 0 time	MDA (µM), 0.5 h	MDA (µM), 4 h	MDA (µM), 8 h	MDA (µM), 24 h
0	N.D.	0.37* (0.085)	9.35* (1.87)	15.67* (2.23)	17.29* (3.12)
1	N.D.	0.31* (0.028)	8.13* (2.05)	13.48* (3.07)	15.36* (2.81)
2	N.D.	0.28*† (0.015)	7.47*† (1.65)	12.45*† (2.18)	14.01*† (3.13)
5	N.D.	0.22*† (0.055)	5.13*† (2.12)	9.62*† (2.66)	10.72*† (3.31)
10	N.D.	0.13*† (0.060)	2.47*† (0.963)	3.59*† (1.01)	4.32*† (1.95)
20	N.D.	0.07*† (0.011)	1.20*† (0.766)	2.01*† (1.21)	2.42*† (1.30)
50	N.D.	0.03*† (0.008)	0.37*† (0.100)	0.52*† (0.121)	0.81*† (0.176)
100	N.D.	0.02*† (0.004)	0.21*† (0.088)	0.43*† (0.094)	0.48*† (0.065)
200	N.D.	0.01*† (0.002)	0.17*† (0.024)	0.26*† (0.036)	0.32*† (0.064)

*Significantly different from respective 0 time ($p < 0.001$).
†Significantly different from corresponding time of control LDL ($p < 0.01$).
N.D.=Not detectable.

TABLE II Effect of pH on the cyanidin-3-*O*-β-glucopyranoside (C-3-G) antioxidant activity. LDL suspensions were incubated with 40 μM Cu^{2+}, for 24 h at 37°C, in 20 mM ammonium acetate, pH 5.0 or 6.0. Levels of lipid peroxidation were estimated by direct HPLC measurements of MDA. Control LDL were incubated in presence of buffer only. Each value is the mean (SD) of five experiments

C-3-G added (μM)	MDA (μM), 0 time, pH 5.0	MDA (μM), 24 h, pH 5.0	MDA (μM), 0 time, pH 6.0	MDA (μM), 24 h, pH 6.0
0	N.D.	16.94* (1.94)	N.D.	17.77* (2.06)
1	N.D.	15.28* (1.78)	N.D.	15.81* (2.28)
2	N.D.	11.04* (1.99)	N.D.	12.00* (1.90)
5	N.D.	9.47* (1.52)	N.D.	10.10* (2.36)
10	N.D.	3.29* (0.73)	N.D.	4.66* (1.31)
20	N.D.	2.33* (0.51)	N.D.	2.70* (0.69)
50	N.D.	1.47* (0.26)	N.D.	1.24* (0.43)
100	N.D.	0.79* (0.10)	N.D.	0.61* (0.09)
200	N.D.	0.53* (0.04)	N.D.	0.44* (0.06)

*Significantly different from respective 0 time ($p < 0.001$).
N.D.=Not detectable.

was already evident at the lowest concentration tested (about 13% inhibition of MDA production). At the highest dose of the antioxidant, the maximal protective effect of C-3-G on LDL oxidation was recorded (98% reduction of MDA generation). Data reported in Table II show the effects of pH changes on the capacity of C-3-G to inhibit LDL oxidation induced by 40 μM Cu^{2+}. Since no statistical differences were observed at the different pH tested (5.0, 6.0 and 7.4), the results clearly indicate that the antioxidant activity of C-3-G is unaffected by pH of the medium, at least in the pH range considered.

In Fig. 2, the dose-response efficacy of C-3-G, resveratrol and ascorbic acid (two other relevant natural antioxidants) on LDL oxidation, induced by incubation with 40 μM Cu^{2+} for 24 h at 37°C, is compared. At any dose tested, C-3-G showed the highest efficiency in preventing LDL oxidation; in particular, the calculated IC_{50} for the different antioxidants were 6.5 μM for C-3-G, 34 μM for resveratrol and 212 μM for ascorbic acid. Figure 3 shows the time-course of copper-induced LDL lipid peroxidation without and with the most effective dose of any of the antioxidant considered. Differently from resveratrol and ascorbic acid, C-3-G seemed to inhibit the initiation phase of lipid peroxidation; this suggests that C-3-G might act either through a possible chelation of copper, which would be no more available for initiating LDL peroxidation, or through its high reducing capacity, which would scavenge the majority of free radicals, thus hindering the attack to double bonds of polyunsaturated lipids of LDL.

In Fig. 4, the inhibitory effect, exerted by different volumes of juices from pigmented and blond oranges on LDL oxidation induced by 40 μM Cu^{2+}, is illustrated. Juice from pigmented oranges (Moro variety) showed significantly higher antioxidant capacity than that of juice from blond oranges, at any volume considered. Since ascorbic acid content was similar (3.2 and 2.8 mmol/l in pigmented and blond juice, respectively), it can be affirmed that the statistical differences concerning inhibition of LDL oxidation between the two orange juices were due to the presence of anthocyanins (90% of which consisted of C-3-G) in the pigmented one. According to our analysis, total anthocyanin content of pigmented juice was 1.2 mM and, consequently, C-3-G was 1.08 mM; due to the dilution effected upon addition to LDL suspension, numbers reported in abscissa nearly correspond to the final micromolar C-3-G concentration.

It is worth recalling that LDL incubated for 24 h at 37°C in presence of buffer only did not undergo to any detectable MDA production, i.e. LDL autooxidation was negligible.

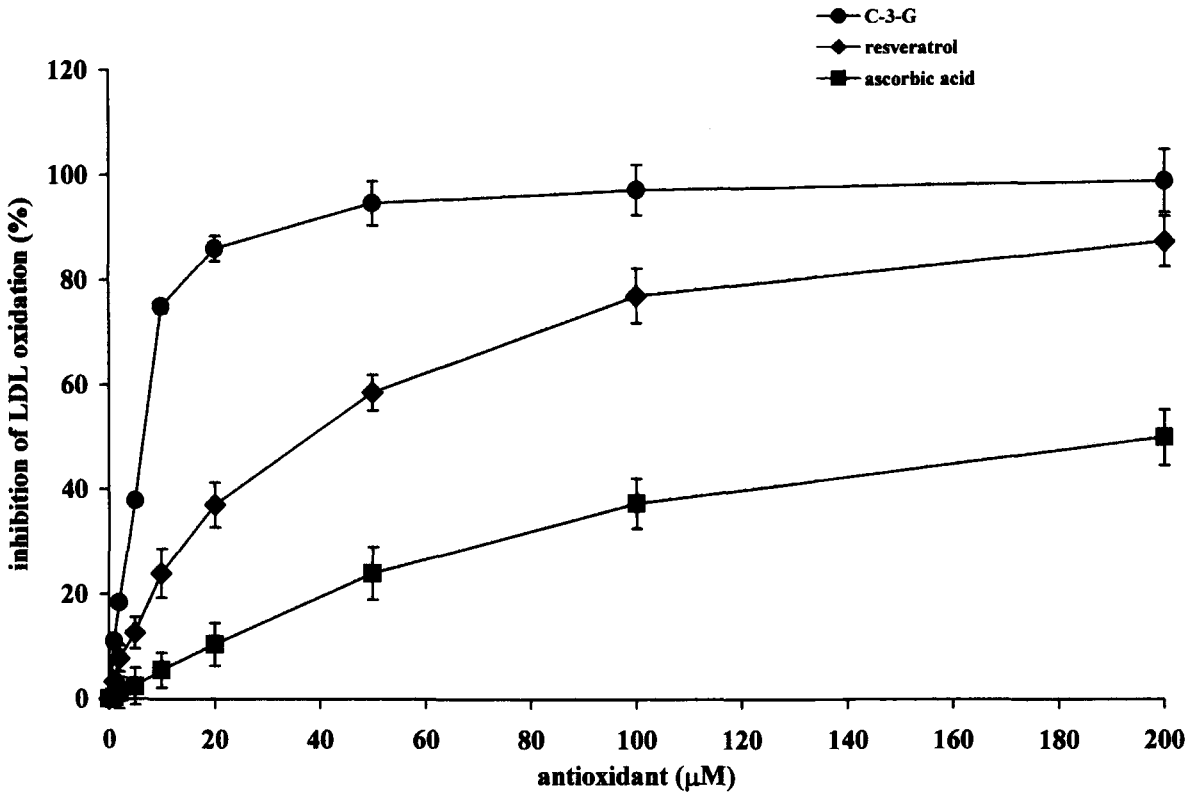

FIGURE 2 Inhibitory effect of increasing concentrations of cyanidin-3-*O*-β-giucopyranoside (C-3-G), resveratrol and ascorbic acid on copper-induced LDL oxidation. LDL were incubated with 40 μM Cu^{2+}, for 24 h at 37°C, in 20 mM ammonium acetate, pH 7.4, supplemented with increasing concentrations of indicated antioxidant. Antioxidant effect was expressed as percent inhibition of lipid peroxidation (estimated by direct HPLC measurements of MDA). Zero percent inhibition was assessed in LDL incubated in presence of buffer only. Each point is the mean of five different experiments. Standard deviations are represented by vertical bars. All C-3-G values were significantly different from corresponding values of other antioxidants.($p < 0.001$).

Interaction of C-3-G with ROS

Figure 5 shows the results concerning the interaction of 100 μM C-3-G with different hydrogen peroxide concentrations. Decrease of C-3-G absorbance at 438 nm was observed when H_2O_2 was added to the incubation mixture; the initial rate of C-3-G oxidation increased linearly by increasing H_2O_2 in the medium and the reaction proceeded (8–10 min) until almost complete C-3-G oxidation occurred (75–95% of the initial concentration with the minimal and maximal H_2O_2 concentration, respectively). The concomitant presence of catalase completely abolished the observed absorbance changes of C-3-G induced by hydrogen peroxide (data not shown).

Figure 6 shows the effect of different C-3-G concentrations on cytochrome c reduction induced by superoxide anions generated by the xanthine–xanthine oxidase system. C-3-G inhibited cytochrome c reduction in a dose-dependent manner with a maximal efficiency at the highest concentration tested (93.6% inhibition) and an approximate IC_{50} of 20 μM.

Figure 7 shows the initial rate of C-3-G oxidation as a function of increasing hydroxyl radicals generated through the Fenton reaction using fixed reduced iron (100 μM) and variable hydrogen peroxide concentrations (0.25, 0.5, 1, 2,

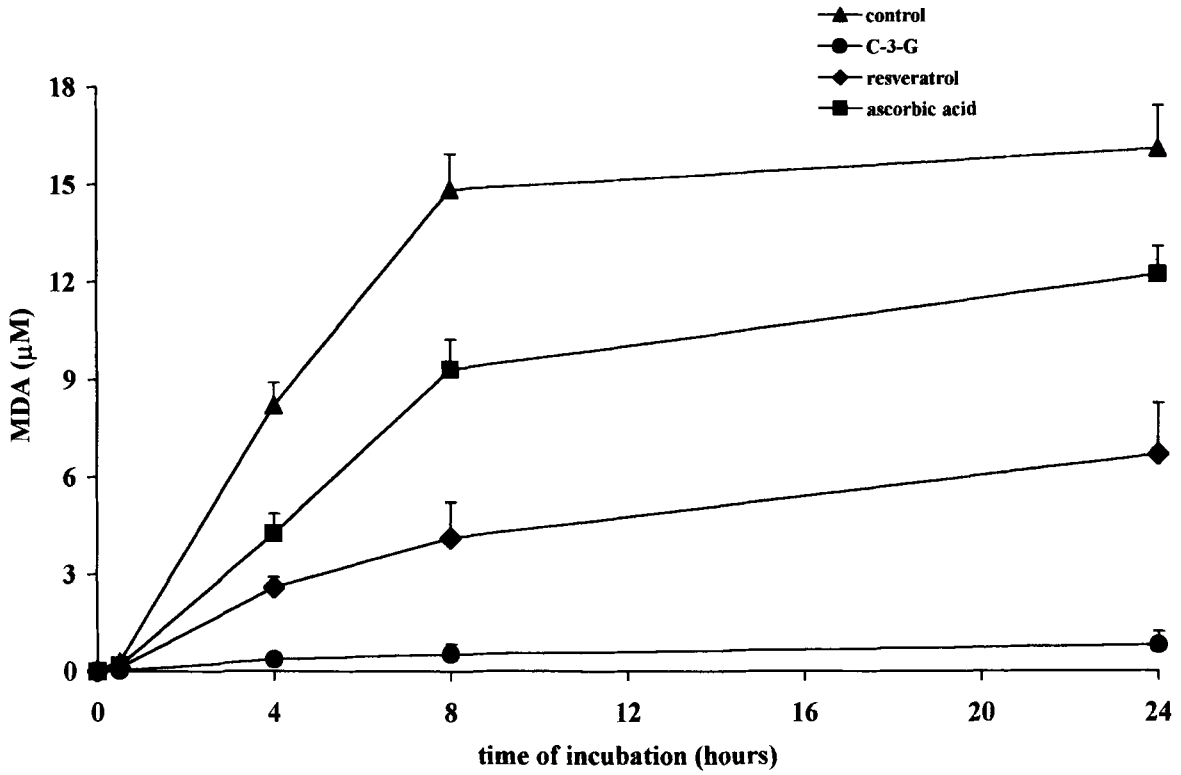

FIGURE 3 Inhibitory effect of same concentrations of cyanidin-3-*O*-β-glucopyranoside (C-3-G), resveratrol and ascorbic acid on the time-course of copper-induced LDL oxidation. LDL were incubated with 40 μM Cu^{2+}, for different times at 37°C, in 20 mM ammonium acetate, pH 7.4, supplemented with 50 μM of the indicated antioxidants. Levels of lipid peroxidation were estimated by direct HPLC measurements of MDA. Control LDL was incubated in the presence of buffer only. Each point is the mean of five different experiments. Standard deviations are represented by vertical bars. All C-3-G values were significantly different from corresponding values either of control LDL or of other antioxidants ($p < 0.001$).

5, 10 and 20 mM). Highest rate was recorded with 20 mM H_2O_2 and, depending on H_2O_2 initial concentration, C-3-G oxidation was completed between 1–3 min. In comparison with data obtained with H_2O_2 only, the initial rate of C-3-G oxidation was more than six times faster when hydroxyl radicals were the oxidizing specie.

Determination of the C-3-G Redox Potential

The redox potential of C-3-G was determined through DC cyclic voltammetry measurements. At a pyrolytic graphite electrode, the DC cyclic voltammograms of C-3-G exhibited a defined electrochemistry for scan rates ranging between 20 and 200 mV/s. Figure 8 shows the DC cyclic voltammogram run at 100 mV/s, in which the cathodic and anodic waves appear similar in shape and magnitude. The peak separation, $\Delta E_p = 194$ mV, is sensibly larger than the theoretical value ($\Delta E_p = 57$ mV at 25°C for a fully reversible one-electron transfer reaction),[19] indicating quasi-reversibility.[20] The calculated redox potential, $E_{1/2} = -405$ mV vs a normal hydrogen electrode (NHE), is more negative than that of any known antioxidant of biochemical relevance at the same pH,[12] including that of ascorbic acid ($E_{1/2} = -174$ mV vs NHE). This demonstrates that C-3-G is a strong reducing agent acting as a powerful antioxidant.

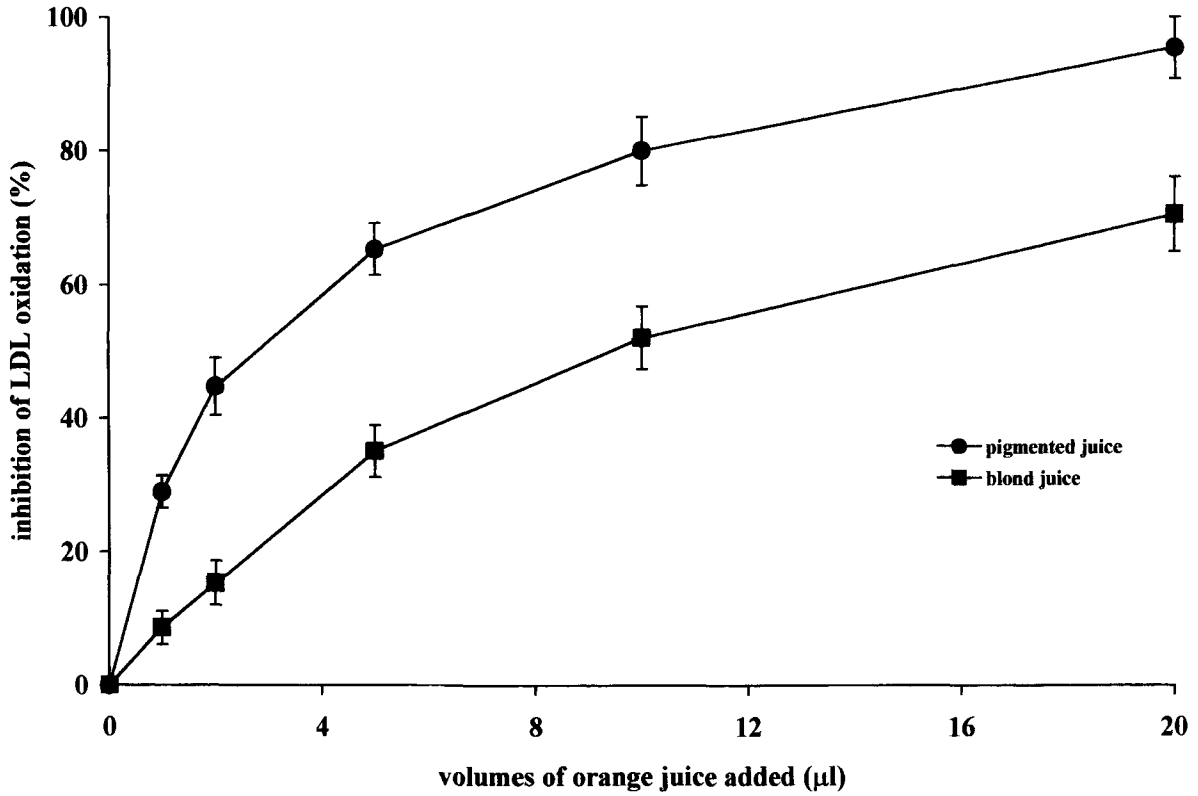

FIGURE 4 *Inhibitory effect of increasing volumes of pigmented and blond orange juice on copper-induced LDL oxidation. LDL were incubated with 40 µM Cu^{2+}, for 24 h at 37°C, in 20 mM ammonium acetate, pH 7.4, supplemented with different volumes of orange juice. Antioxidant effect was expressed as percent inhibition of lipid peroxidation (estimated by direct HPLC measurements of MDA). Zero percent inhibition was assessed in LDL incubated in presence of buffer only. Each point is the mean of five different experiments. Standard deviations are represented by vertical bars. All pigmented juice values were significantly different from corresponding values of blond juice ($p < 0.001$).*

DISCUSSION

Several explanations of anthocyanin antioxidant capacity were suggested in the past, such as the ability to scavenge alkoxyl and peroxyl radicals,[22] the metal chelation activity,[14,23] the possibility of binding proteins.[24] Anthocyanin effects may be mediated, in emulsified systems, by phase distribution[25] and interfacial phenomena[26] and are, however, dependent on the different chemical structure of the various anthocyanins. It has also been shown that the substitution pattern of OH groups on the three rings of anthocyanins (mainly that on the B ring) may influence their respective antioxidant capacities,[4,10] even if some unclear results do not allow an easy generalization useful for definitively correlating chemical structure and antioxidant function of anthocyanins.[4,10,17] Our results, performed with the aim of characterizing the activity of the most abundant anthocyanin of pigmented oranges, i.e. C-3-G, demonstrated that C-3-G, in addition to the aforementioned mechanisms, can elicit its powerful antioxidant action by directly interacting with radical and non-radical oxidizing species present in the medium. The experiments conducted with the Cu^{2+}-oxidized LDL, clearly showed that C-3-G has no prooxidant but only antioxidant properties; in fact a dose-dependent protection of LDL

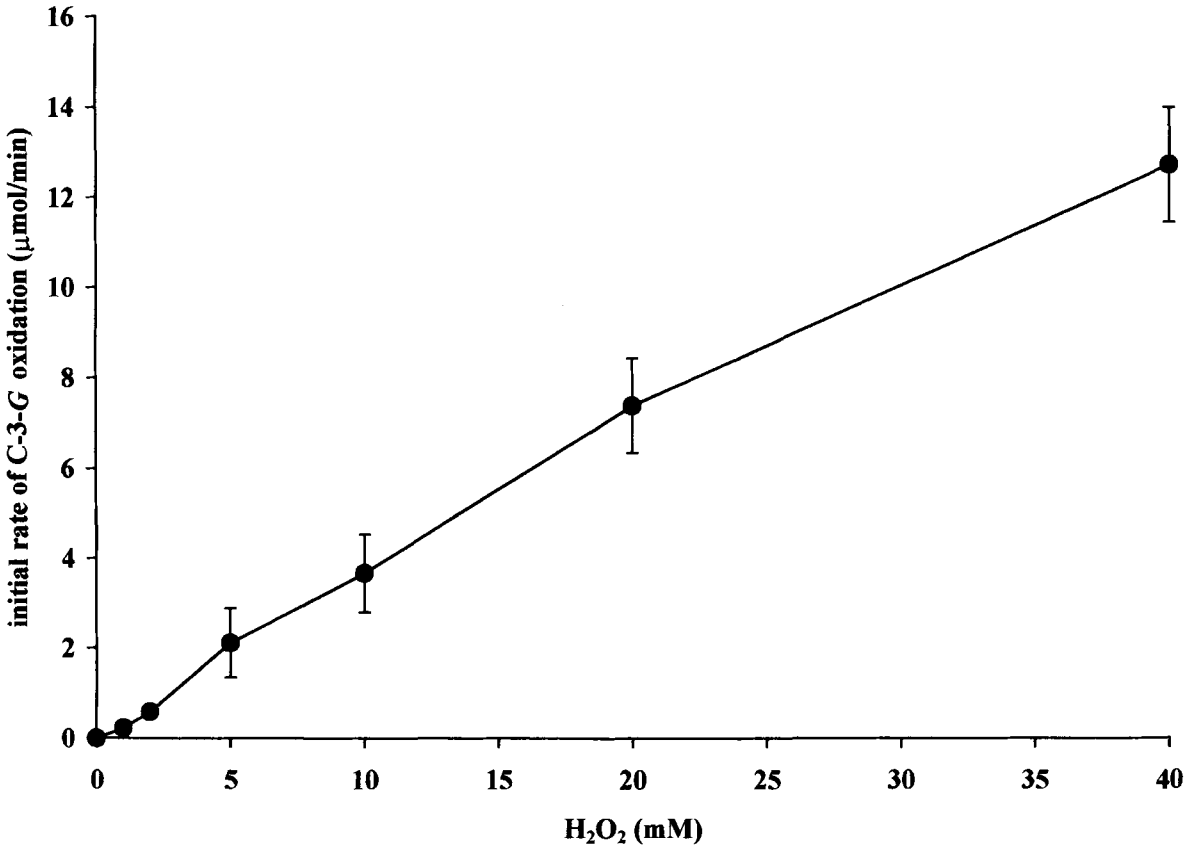

FIGURE 5 Hydrogen peroxide-induced cyanidin-3-*O*-β-glucopyranoside (C-3-G) oxidation. C-3-G (100 μM) was incubated with increasing H_2O_2 concentrations at 37°C, in 20 mM phosphate buffer, pH 7.0. Initial rate of C-3-G oxidation was determined spectrophotometrically at 438 nm wavelength. Each point is the mean of four different experiments. Standard deviations are represented by vertical bars.

oxidation as a function of increasing C-3-G was observed starting from the lowest concentration tested (1 μM = 13% inhibition of LDL oxidation). From the data shown in Fig. 2, in which 10 μM and 20 μM C-3-G exerted a protection of LDL oxidation by 75 and 86%, respectively, it seems difficult to sustain that such an effect is only due to C-3-G metal chelation. If this were true, it should be admitted that stoichiometry of metal binding to C-3-G is about 3:1. According to previous observations, chelation of Cu^{2+} is expected to occur *via* the interaction with the two OH groups of the anthocyanin B ring,[4] therefore implying a 1:1 binding stoichiometry. This might produce a maximal protection of 25 and 50% with 10 and 20 μM C-3-G, respectively. In general, the possibility that metal chelation may be responsible for the antioxidant C-3-G activity is even less convincing because such a mechanism requires, at neutral pH too, a high OH dissociation of anthocyanins, whereas these compounds are known to act as weak acids. Data reported in the present manuscript, referring to the C-3-G capacity to protect copper-induced LDL oxidation at pH 5.0 and 6.0, strongly suggest that metal chelation plays a minor role in the overall antioxidant activity of C-3-G. Since no change in the antioxidant effect was recorded as pH of the medium was decreased from 7.4 to 5.0 (which excludes titration of the OH groups), it is

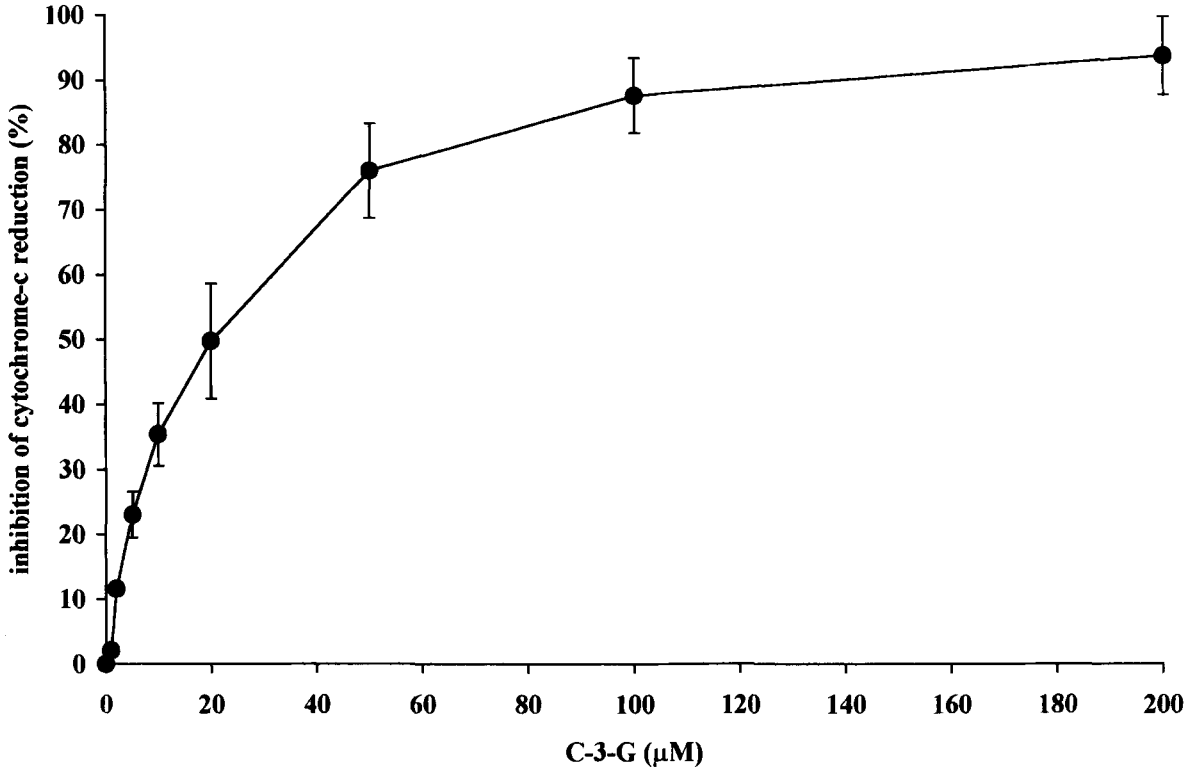

FIGURE 6 Dose-response inhibition of superoxide production by cyanidin-3-*O*-β-glucopyranoside (C-3-G). Increasing C-3-G concentrations were incubated at 37°C with 300 μM xanthine, 100 μM cytochrome c, 0.07 U/ml xanthine oxidase, in 20 mM phosphate buffer, pH 7.0. Inhibition of cytochrome c oxidation was determined spectrophotometrically at 550 nm wavelength. Each point is the mean of four different experiments. Standard deviations are represented by vertical bars.

hard to hypothesize that C-3-G may bind, with its OH groups undissociated or partially dissociated, such a metal quantity able to produce a so remarkable inhibition of LDL oxidation.

Our results also demonstrated that C-3-G antioxidant effect is much more pronounced than that of two other naturally occurring antioxidants (resveratrol and ascorbic acid), at any concentration tested. In fact, even at the highest dose used both resveratrol and ascorbic acid produced significantly lower protective effect on LDL oxidation than C-3-G. Consequently, the order of magnitude in the IC_{50} values of resveratrol and ascorbic acid were 5.2 and 32.6 times higher, respectively, than that of C-3-G.

According to our data, carried out to elucidate the mechanism of C-3-G antioxidant activity, it is possible to affirm that this anthocyanin is a potent direct scavenger of ROS. The reactivity of C-3-G towards superoxide anions, as revealed by the experiments with the xanthine–xanthine oxidase superoxide-generating system (Fig. 6), is particularly interesting because of the biological role of $^{-}O_2$ in the development of the so called oxidative stress. In fact, it is generally accepted that superoxides, produced from various cellular sources under physiological and various pathological conditions,[27–29] represent the starting point for generating more dangerous radicals. This phenomenon, occurring in situations of increased oxidative stress, generates relevant amounts of highly toxic ROS which are responsible for the irreversible damage of biologically fundamental macromolecules.[30–32] Since C-3-G

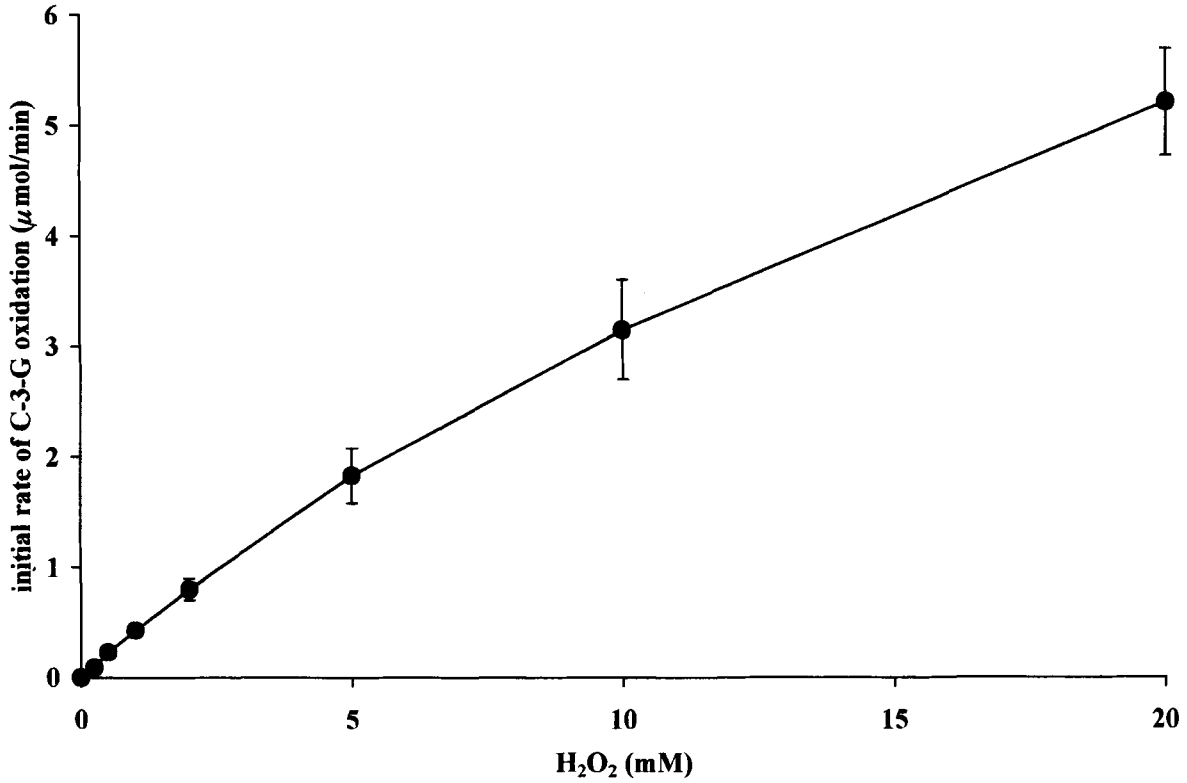

FIGURE 7 Hydroxyl radical-induced cyanidin-3-*O*-β-glucopyranoside (C-3-G) oxidation. Fe^{2+} (100 μM) was complexed by pre-incubation at 37°C in 20 mM phosphate buffer, pH 7.0. C-3-G (100 μM) was supplemented before the addition of variable H_2O_2 concentrations. Initial rate of C-3-G oxidation was determined spectrophotometrically at 438 nm wavelength. Each point is the mean of four different experiments. Standard deviations are represented by vertical bars.

showed a remarkable scavenger activity either towards hydroxyl radicals and hydrogen peroxide, the assumption that this anthocyanin act as a strong reducing agent appears quite reasonable. The experiments conducted by DC cyclic voltammetry allowed to determine the C-3-G redox potential ($E_{1/2} = -405$ mV vs NHE); this value is more negative than the majority of molecules with biological relevance,[21] thereby indicating that C-3-G should occupy a pre-eminent position in the hierarchy of natural scavenger compounds, also in view of its diffusion in dietary plants. In this light, it is worth underlining results of experiments on copper-induced LDL oxidation in which the relative potency of pigmented and blond orange juices was compared. Pigmented orange juice, which contains notable levels of anthocyanins mainly represented by C-3-G,[3,16,17] produced significantly higher inhibition of LDL oxidation than blond orange juice. On the basis of their mean content (1–1.5 mM/l pigmented orange juice), an intake of 2.5 pigmented oranges (about 200 ml of juice) would furnish 200–300 μmol of C-3-G which represent 4 times and 1/9 of the minimal and maximal calculated total flavonoid intake in human beings, respectively.[33,34] It was recently demonstrated that C-3-G is partly absorbed by the intestine and is found in plasma and tissues,[35,36] although it is not yet clear whether it is present in its native form only or in form of its metabolites too (the corresponding aglycone cyanidin and protocatechuic acid). It should be outlined that the eventual metabolites

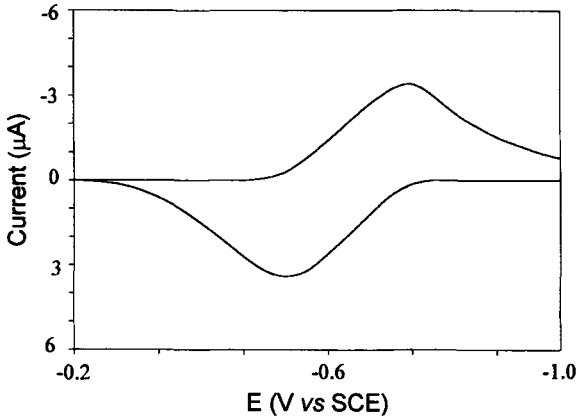

FIGURE 8 Direct current (DC) cyclic voltammogram of cyanidin-3-*O*-β-glucopyranoside (C-3-G). DC cyclic voltammetry of C-3-G (1.5 mg/ml) was carried out at 25°C at a pyrolitic electrode, in the potential range −0.2 to − 1.0 mV vs SCE. Measurements were effected in 50 mM phosphate buffer, pH 7.0, containing 50 mM NaCl as supporting electrolyte. The voltammogram illustrated is subtracted of the background current.

deriving from C-3-G intake still have potent antioxidant properties, similar to those of the parental molecule.[35] Therefore, pigmented oranges should be considered as one of the primary alimentary sources for improving, through C-3-G intake, resistance of organism to oxidative stress insults. It should be recalled that pigmented oranges, mainly growing in Sicily and representing over 70% of total cultivar in Italy (which is the major orange producer among European countries), are a fundamental food in the so called "Mediterranean diet".

In conclusion, data reported in the present paper add new information on the antioxidant properties of the most abundant anthocyanin of pigmented oranges. The remarkable scavenger efficacy, due to the very negative redox potential, renders C-3-G a relevant antioxidant molecule whose dietary intake should be strongly recommended. In this light, the presence of high levels of C-3-G in pigmented oranges (Moro, Sanguinello and Tarocco cultivar) support the hypothesis that a regular pigmented orange (or juice) intake may greatly contribute to increase the individual antioxidant capacity, thus ameliorating the defense mechanism against ROS-mediated cell and tissue injury.

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