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ARTICLE

Protection of Fibroblasts (NIH-3T3) against Oxidative Damage by Cyanidin-3-rhamnoglucoside Isolated from Fig Fruits (*Ficus carica* L.)

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Anthocyanins, plant secondary metabolites, have been recognized for their health-promoting properties when consumed by humans. In this study, the antioxidant properties of a major anthocyanin in fresh fig fruits, cyanidin-3-rhamnoglucoside (C3R), were evaluated by various assays in vitro and correlated with the protection afforded by C3R to cultured NIH-3T3 fibroblast cells. C3R inhibited lipid peroxidation from producing peroxy radicals ($ROO^{\bullet}$) and MDA in a dose-dependent manner, and a high calculated stoichiometric coefficient [*n*] for peroxy radicals was demonstrated. In addition to its scavenging of reactive oxygen species (ROS), C3R showed a strong chelating activity toward the Fe^{2+} ion. Finally, pretreatment with C3R inhibited proapoptotic processes that were initiated by the oxidation of lysosome membranes in fibroblast cells. The high antioxidant potential, with several modes of action of purified C3R, may contribute to health benefits gained by the consumption of fresh fig fruits.

KEYWORDS: Fig fruits; cyanidin-3-rhamnoglucoside (C3R); ROS; NIH-3T3 fibroblasts; Fe^{2+} chelation; linoleic acid oxidation; lysosome membrane stability

INTRODUCTION

Flavonoids are a large group of compounds naturally produced from plants as part of their attracting system and their diverse defense mechanisms against stresses of different origins (*1*). During the past decades, scientific evidence has indicated that adult human diets rich in flavonoids lead to significantly decreased serum concentrations of total cholesterol, low-density lipoproteins (LDL), and triglycerides, as well as a reduced incidence of cardiovascular diseases (*2*, *3*) and osteoporosis (*4*). Recent studies demonstrate that antioxidant-rich diets can not only positively impact health but also reduce age-related cognitive decline and improve learning and memory (*5*, *6*). Anthocyanins are one of the main classes of flavonoids (*1*). Some have suggested that the molecular entities of anthocyanins are most likely responsible for what has been described as the "French Paradox" (*7*). Anthocyanins exhibit antioxidant activities and a variety of pharmacological properties, such as chemoprevention of cancer, heart, and inflammatory diseases (*8*−*11*). Of particular interest are the flavonoid anthocyanins, which impart red and blue colors to berries, grapes, red wine, and other fruits such as figs.

The fig fruit (*Ficus carica* L.) has been a characteristic component in the health-promoting Mediterranean diet for millennia. Today, fig is an important crop worldwide for dry and fresh consumption (*12*, *13*). Recently, we described for the first time the isolation and identification of cyanidin-3-rhamnoglucoside (cyanidin-3-rutinoside; C3R, see **Figure 1**), the main anthocyanin in the skin of fig fruits, accounting for 95% of the total anthocyanins (*14*). This natural compound is also distributed in several other fruits and vegetables, such as fresh olives, mulberries, cherries, acai palm berries (*Euterpe oleracea* Mart.), and black currant (*Ribes nigrum* L.) (*15*−*19*). The present study was designed to characterize modes of antioxidant action of C3R purified from fig skin extract, as previously described (*14*). Within this framework, we have employed several antioxidant assays to determine the potency of C3R in vitro and determined its capacity to protect cells from oxidative damage. To this end, the widely used nonmalignant mouse fibroblast cell line NIH-3T3 was used as a model system to study the protective effect on cells.

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MATERIALS AND METHODS

Materials. Linoleic acid, malondialdehyde (MDA), 2′,7′- dichlorofloro-fluorescin diacetate (DCFH-DA), ferrozine, diethylenetriaminepentaacetic acid (DTPA), acridine orange (AO), [(2,2′-azo-bis (2-amidinopropane)] dihydrochloride (AAPH), *N*-methyl-2-phenylindole, methanesulfonic acid, *trans*-resveratrol, ferulic acid, citric acid, hydrogen peroxide, and ferrous sulfate were obtained from Sigma Chemical, USA. Fibroblast cells (NIH-3T3) were obtained from the American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEN), fetal calf serum (FCS), and penicillin−streptomycin were obtained from Biological Industries Ltd., Beit - Haemek, Israel.

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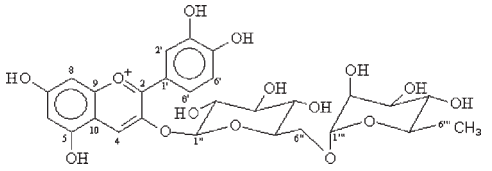

Figure 1. Chemical structure of cyanidin-3-rhamnoglucoside (C3R), the main anthocyanin in fig fruits (*Ficus carica* L.).

Isolation of Cyanidine-3-rhamnoglucoside (C3R) from Fresh Mission Fruits. Isolation and purification of C3R from the fresh skin of the Mission variety (dark fig fruits) was previously described (*14*). Briefly, fruit skins were seporated and homogenized in a Waring blender containing chilled acidified methanol (0.1% acetic acid v/v) to extract anthocyanins. The slurry was centrifuged, and the anthocyanin-containing supernatant fluid was decanted. The extraction procedure was repeated four times, and supernatant fluids were pooled and evaporated under vacuum to dryness. The residue was then redissolved in acidified methanol and passed through a 0.2 μM regenerated-cellulose filter, and C3R was purified using reversed phase liquid chromatography (RP-LC) as described previously (*14*).

Determination of Antioxidant Potential. *Inhibition of the Auto-Oxidation of Linoleic Acid.* Linoleic acid (C18:2, $n-6$) was prepared as described previously (*20*), and the conjugated-dienes assay was performed as described previously (*21*) with minor modifications. The peroxidation of linoleic acid (0.3 μM) was initiated by thermal degradation at physiological temperature, 37 °C, by a water-soluble azo initiator AAPH ([(2,2′-azo-bis (2-amidinopropane)] dihydrochloride; 0.2 mM) and in the presence of graded concentrations (0 to 2.5 μM) of C3R. The rate of oxidation at 37 °C was monitored by recording the increase in absorption by conjugated diene hydroperoxides at 234 nm. Assays were conducted in triplicate in PBS buffer (50 mM, pH 7.4) and compared with a separate AAPH-free control for any spontaneous oxidation. AAPH has a relatively high absorbance below 260 nm, which changes as the compound decomposes. Therefore, its absorbance, measured in a separate cuvette in the absence of linoleic acid, was subtracted from each experimental point. The inhibition time (T_{inh}) was estimated with Microsoft Excel software as the point of intersection between the tangents to the inhibition and propagation phase curves, under precise oxidation conditions (*21*). T_{inh} versus the C3R concentration is another antioxidant activity index. The slope of the curve is represented by S and S'. A stoichiometric coefficient n representing the number of peroxyl radicals ($ROO^{\bullet}$) trapped by the antioxidant molecule can be extracted from the equation: $Ri = n \times [\text{antioxidant}]/T_{inh}$ (*21*, *22*).

Determination of Malondialdehyde (MDA). Determination of MDA was based on the reaction of the chromogenic reagent, *N*-methyl-2-phenylindole, with MDA and 4-hydroxyalkenals (4-HNE) at 39 °C and monitored at 586 nm (*23*). First, 80 μL of linoleic acid was dissolved with 240 μL of phosphate buffer (50 mM, pH 7.4) containing graded concentrations (0−40 μM) of C3R. The solution was diluted to a final volume of 0.25 mL, and 25 mM AAPH was added last to initiate the reaction. Following incubation at 39 °C for 1 h, 40 μL aliquots were taken to determine MDA, in 96-well plates. Finally, 130 μL of *N*-methyl-2-phenylindole (10 mM in acetonitrile) and 30 μL of methanesulfonic acid (15.4 M) were added, and absorbance was recorded at 586 nm using a spectrophotometer (Tecan). The assay was conducted in triplicate.

Chelation of Ferrous Ion (Fe^{2+}). The chelation of ferrous ion by C3R was determined as previously described (*24*) with some modifications. $FeSO_4$ (70 μM) was mixed with ferrozine (140 μM) and with graded concentrations (0 to 12.8 μM) of C3R in doubly distilled water (DDW) and agitated for 10 min at room temperature. The absorbance of the resulting colored Fe^{2+}−ferrozine complex in the presence or absence of C3R was measured at 562 nm. DTPA, a specific chelator for Fe^{2+}, was used as a positive control. Citric acid, *trans*-resveratrol, and ferulic acid (10−80 μM) were used as negative controls. The ferrous-chelation capacity of C3R was calculated as follows:

$$\text{chelating}\% = [(1 - (\text{absorbance of sample at 562nm})/(\text{absorbance of positive control at 562nm})] \times 100$$

Determination of ROS in Fibroblast Cells (NIH-3T3). The level of ROS in the cells was assayed according to Hait-Darshan et al., with some modifications (*25*). Nonfluorescent 2′,7′- dichloroflorofluorescin diacetate (DCFH-DA) was used for monitoring the ROS with a spectrofluorometer (wavelength 485/535 nm) capable of reading microplates. NIH-3T3 fibroblasts cells were maintained in Dulbecco's modified Eagle's containing 10% fetal calf serum (FCS) and 1% penicillin−streptomycin. Cells were counted and plated (1×10^4 cells per wall) in 96-well tissue culture plates (flat-bottomed). Cells were maintained at 37 °C and 5% CO_2 in a humid environment for 24 h. C3R at the range of 0 to 10.2 μM (in PBS) was added to the wells containing the cells. A stock solution of DCFH-DA (5.13 mM) was prepared as described previously (*25*). Plates were incubated for 30 min at 37 °C with 5.13 μM DCFH-DA in each well. Oxidation was initiated by the addition of $FeSO_4$ to a final concentration of 80 μM and then read with a spectrofluorometer (wavelength 485/530 nm). Nonfluorescent DCFH-DA permeates cell membranes and is deacetylated by intracellular esterases into dichlorofluorescein, which is reactive with hydrogen peroxide to produce the fluorescent compound, dichlorofluorescein (DCF) (*26*).

Lysosomal Membrane Stability Assay. Fibroblast cells (NIH-3T3) at a density of 4×10^4 cells per well were suspended in complete medium (DMEN) for 24 h. Cells were washed twice in PBS (50 mM, pH 7.5) and exposed to oxidative stress for 10 min by adding 75 μM H_2O_2 at 37 °C. After washing twice with PBS, cells were exposed to 15 μM of the lysosomotropic weak base, acridine orange, for 20 min at 37 °C, followed by several rinses in PBS. All steps were carried out in the dark. To test the protective effect of C3R, NIH-3T3 cells were incubated with 100 μM H_2O_2 and 20 μM C3R for 10 min at 37 °C. Thereafter, the cells were treated with acridine orange (AO), as described above.

Calculation of IC_{50} Values of C3R. The IC_{50} value of C3R was determined by linear regression and estimated using the fitted line as follows:

$$Y = aX + b$$

$$IC_{50} = (0.5 - b)/a$$

Statistical Analysis. All determinations ere based on at least three independent replicate samples. Results were analyzed by JMP IN statistical discovery software using one-way variance analysis (ANOVA). When a significant difference was obtained ($p < 0.05$), the Tukey-Kremer HSD test was used to compare each pair of means.

RESULTS

Inhibition of the Peroxidation of Linoleic Acid. To initially determine the antioxidant protection afforded by C3R, we measured the rate of production of conjugated dienes through the oxidation of linoleic acid. In the absence of the radical initiator, the rate of spontaneous oxidation at 37 °C was negligible. Once AAPH was added to the assay solution at 37 °C, conjugated dienes were produced at a constant rate, allowing the determination of inhibition time (T_{inh}) for each C3R concentration (0, 0.3, 1.0, and 2.5 μM). The T_{inh} values were 18, 50, 120, and 210 min, respectively (**Figure 2**A). Peroxidation of linoleic acid or its esters gives different hydroperoxides depending on the reaction conditions (*27*). Hydroperoxide substitution at the C-9 or C-13 positions produces either trans- or cis,trans-conjugated dienes which are the major products in the absence of antioxidants or in the presence of a low level of antioxidants. These conjugated dienes are formed from the rapid β-scission of the primarily formed bis-allylic 11-peroxyl radical (*28*). The present experiment included very low levels of antioxidants (micromolar concentrations of C3R); hence, the production of the nonconjugated 11-hydroperoxide should be negligible, and the conjugated hydroperoxides were the predominant products. The latter showed characteristic ultraviolet absorption at 234 nm that was used to monitor the formation of the total hydroperoxides during peroxidation.

In all cases, the reaction reached termination of the inhibitory activity, which was followed by the production of oxidation

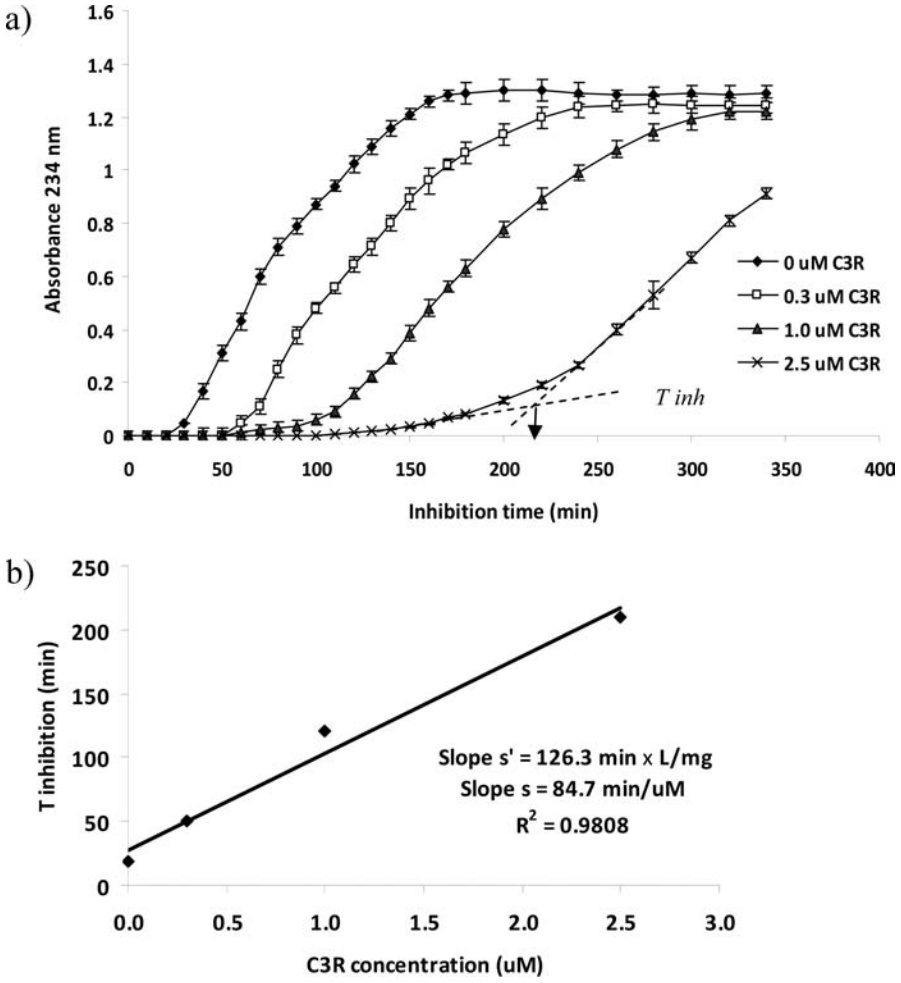

Figure 2. (**A**) Formation of conjugated diene hydroperoxides during the oxidation of linoleic acid (3.0 μM) at pH 7.4 and 37 °C, initiated with 0.2 mM AAPH. The inhibition time (T_{inh}) was estimated with Microsoft Excel software as the point of intersection between the tangents to the inhibition and propagation phase curves, under precise oxidation conditions. The T_{inh} values were 18, 50, 120, and 210 min for 0, 0.3, 1.0, and 2.5 μM of C3R, respectively. Values represent the mean ± SD of three independent experiments. (**B**) Relationship between the concentration of C3R and the oxidation inhibition time (T_{inh}). Inhibition time (18, 50, 120, and 210 min) was determined for each C3R concentration (0, 0.3, 1.0, and 2.5 μM) respectively. Measured inhibition time (T_{inh}) was directly proportional to the concentration of the antioxidant in the tested range. *S* or *S'* is suggested as an index for the antioxidant capacity of molecules.

products at the same rate as in the absence of inhibitor (not shown). This suggests the complete consumption of the inhibitor. The slope of the curve (*S* or *S'*) representing the T_{inh} versus the antioxidant concentration, shown in **Figure 2**B, was previously suggested as an index for antioxidant capacity of molecules (*22*). A stoichiometric coefficient *n* representing the number of peroxyl radicals ($ROO^{\bullet}$) trapped by the antioxidant molecule can be extracted from these data. The rate of radical initiation (*Ri*) under our assay conditions is roughly 1.3×10^{-9} M/s (*22*). Using the equation $Ri = n \times [\text{antioxidant}]/T_{inh}$, resulted in an *n* value for C3R of greater than 80.

Production of MDA. Determination of MDA is yet another measure for the antioxidant capacity that supports the inhibition of lipid oxidation as a mode of action of natural antioxidants. The IC_{50} determined for the inhibition of the production of MDA by C3R was 6.0 μM (**Figure 3**). These results indicate that C3R can delay the oxidation of polyunsaturated fatty acids by the hindrance of primary and late lipid peroxidation products. Among the lipid peroxidation products used for antioxidant assays, MDA has been most widely used to evaluate the antioxidant activity of chemical(s) in lipid peroxidation systems. In particular, MDA is a useful biomarker to investigate the final stage of lipid peroxidation (*29*).

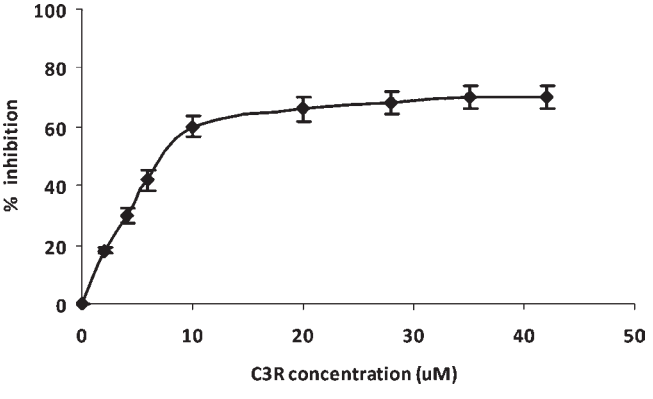

Figure 3. Effect of C3R concentration on malondialdhyde (MDA) and 4- hydroxynonenal (4-HNE) formation in the linoleic acid chain cleavage products (an index of lipid peroxidation). Percentage of inhibition was determined by the reaction with the cheromogenic reagent *N*-methyl-2-phenylindole. The chromophore absorbance was measured at 586 nm. IC_{50} = 6.0 μM C3R. Values represent the mean ± SD of three independent experiments.

Chelation of Ferrous Ion (Fe^{2+}). Transition metals such as copper and iron are known to aggravate oxidative stress. Ferrous

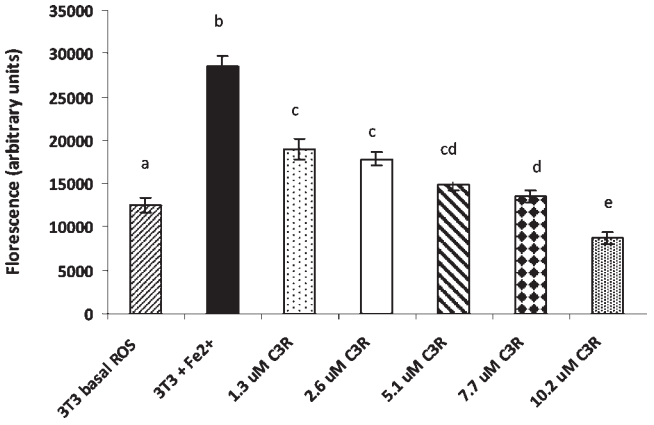

Figure 4. Relationship between ROS production and cyanidin-3-rhamnoglucoside (C3R) concentration in fibroblast cells (NIH-3T3). Oxidation was initiated by Fe^{2+} in a final concentration of 80 μM. Fe^{2+} was added in the presence of C3R. 2′,7′-Dichloroflorofluorescin-diacetate (DCFH-DA) was used to determine ROS levels. IC_{50} = 7.6 μM C3R. Values represent the mean ± SD of three independent experiments. Different letters indicate significant differences ($p < 0.05$).

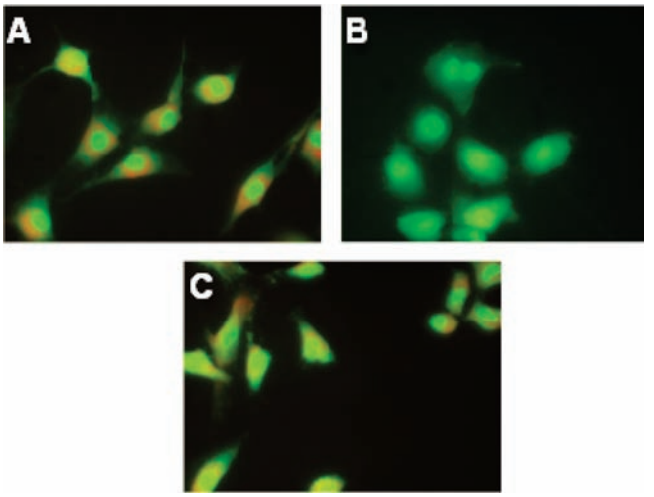

Figure 5. Influence of C3R on the integrity of lysosome membranes in fibroblast cells (NIH/3T3). (**A**) Fibroblast (NIH-3T3) control. (**B**) Oxidative stress induced by 75 μM H_2O_2. (**C**) Pretreatment with 20 μM cyanidin-3-rhamnoglucoside (C3R) and then oxidation with 75 μM H_2O_2. AO was used to monitor the integrity of lysosome membranes and preapoptosis.

chelation may contribute to the antioxidative capacity of a compound by retarding metal-catalyzed oxidation (*30*). Here, the IC_{50} values for the chelation of Fe^{2+} by C3R, diethylenetriaminepentaacetic acid (DTPA), a specific chelator of Fe^{2+}, and citric acid, a general metal ion chelator, were found to be 13.0 ± 0.9 μM for C3R, 25.0 ± 1.6 μM for DTPA, and 10.5 ± 0.8 mM for citric acid. *trans*-Resveratrol and ferulic acid served as negative controls.

Inhibition of ROS in Growing Fibroblasts by Cyanidin-3-rhamnoglucoside (C3R). We next used the DCFH-DA fluorescent assay for the production of ROS by NIH-3T3 fibroblasts in response to metals such as iron to evaluate the potential protection afforded by C3R (**Figure 4**). The effect of C3R on oxidation products was measured 15 min after exposure to ferrous iron (Fe^{2+}). Exposing fibroblasts to Fe^{2+} in the absence of C3R resulted in a significant increase in ROS formation. However, adding C3R (1.3 to 10.2 μM) to the incubation medium prior to exposure to Fe^{2+} significantly inhibited the oxidative response of the cells, in a dose-dependent manner. Maximal efficiency was achieved at the highest concentration tested, 10.2 μM. (70% inhibition), with an IC_{50} of 7.6 μM.

Lysosomal Membrane Stability Assay. Retention of the proton gradient across the membranes of the acidic vacuolar compartment was studied by adding the lysosomotrophic weak base AO to NIH-3T3 cells. Upon entering the acidic internal environment of an acidified late endosome or a lysosome, AO is protonized (charged) and thus trapped, and therefore accumulates. When excited by blue light, highly concentrated AO in lysosomes emits a bright red granular fluorescence (**Figure 5A**), whereas uncharged and diluted AO in the cytosol emits a diffuse greenish fluorescence (*31*). H_2O_2 exposure caused relocalization of AO from the lysosomes to the cytosol and later out of the cell, as indicated by a decline in red fluorescence and an increase in green fluorescence (**Figure 5B**). Pretreatment with 20 μM cyanidin-3-rhamnoglucoside (C3R) before oxidation resulted in apparent protection of the lysosomes against H_2O_2 oxidation and acridine orange relocalization (**Figure 5C**).

DISCUSSION

Anthocyanins are continuously shown to be promising dietary health promoting polyphenolics in many biological systems, due to their potent antioxidant capacity, and absorption in model animals and humans (*1*, *5*, *41*). In a previous study, we showed C3R to be the main anthocyanin in figs, contributing 36% of the total antioxidant capacity of dark fruits (*14*). Here, several modes of antioxidant action of C3R were assayed in vitro showing high capacity and providing protection to fibroblast cells in culture, suggesting that C3R is one of the more promising dietary anthocyanins in acting as an antioxidant in biological systems.

Oxidation of polyunsaturated fatty acids in cell membranes by reactive oxygen species such as $O_2^{\bullet -}$, $^{\bullet}OH$, $^1O_2$, and lipid peroxides has been suggested as one of the primary events in oxidative cellular damage (*29*, *32*). Reactive lipid alkoxyl radicals can be produced from lipid hydroperoxides through a one-electron reduction and then act as chain initiators of lipid peroxidation (*33*). The parameters derived from the kinetic curves indicate that C3R inhibits linoleic acid peroxidation in a concentration-dependent manner; for C3R, inhibition was at least 9-fold higher (n_{C3R} = 80) than that of other antioxidants assayed using the same analysis (*21*, *31*): $n_{quercitin}$ = 8.58, $n_{catechin}$ = 6.12, $n_{caffeic\ acid}$ = 5.07, $n_{vitamin\ E}$ = 3.22, $n_{ferulic\ acid}$ = 2.95, $n_{curcumin}$ = 2.7, n_{trolox} = 2.00, $n_{glutathione}$ = 0.56, and $n_{vitamin\ C}$ = 0.53. These results suggest that C3R is more efficient than other polyphenols in scavenging free radicals such as peroxyl ($ROO^{\bullet}$), as indicated by inhibition periods that are longer than the reported values for these compounds (*21*, *34*). Kaneko et al. have shown that anthocyanidins such as cyanidin, pelargonidin, and delphinidin have a protective effect against the toxicity induced by linoleic acid hydroperoxide in cultured human fetal lung fibroblasts (TIG-7) (*33*). In addition, Kahkonen et al. (*35*) have shown that anthocyanidins and their glycosidic forms acted as antioxidants in lipid environments in vitro. Together with our results, it is suggested that the prevention of lipid peroxidation through the scavenging of lipid hydroperoxides is one pf the routes by which anthocyanins protect cells from oxidative damage.

It has been hypothesized that toxicity caused by oxidation is due to secondary products such as MDA rather than reactive oxygen species because of MDA mutagenicity and its ability to form an adduct with DNA (*36*). MDA reacts both irreversibly and reversibly with proteins, phospholipids, collagen, and DNA, leading to profound effects (*37*). Indeed, it was shown here that

C3R can reduce MDA concentrations in addition to scavenging free radicals such as peroxyl ($ROO^{\bullet}$); these results demonstrated that C3R can delay the oxidation of polyunsaturated fatty acids by hindrance of primary and of late lipid peroxidation products. Other anthocyanins such as cynidin, pelargonidin, keracyanin, and callistephin were also shown to inhibit MDA formation in oxidized calf thymus DNA (*36*).

Chelation of metal ions is one of the suggested modes of actions of antioxidants in living systems, and the ability of a substance to chelate iron can be a valuable antioxidant property. In nature, iron can be found as either ferrous (Fe^{2+}) or ferric (Fe^{3+}) ions, with the latter form predominating in foods. Ferrous chelation may be one more route to inhibit oxidative stress or damage by retarding metal-catalyzed oxidation (*30*). Studies have shown that metal chelators and chain-breaking antioxidants such as phenolic compounds, which are able to scavenge peroxyl radicals, can inhibit lipoprotein oxidation (*38*−*40*). The chelating activity of C3R on Fe^{2+}, as observed here, may play an important role in its apparent antioxidant activity. The structural features of C3R can explain its potent function. C3R has a catachol group on its B ring, which is responsible for trace metal chelating. Free iron and copper are potential enhancers of reactive oxygen species formation, as exemplified by the reduction of H_2O_2 with the generation of hydroxyl free radicals (*40*).

Anthocyanins, like other flavonoids, are thought to localize on the polar surfaces of phospholipid bilayers in a region suitable for scavenging aqueous oxygen radicals and lipophilic radicals incorporated into the membranes (*33*). Anthocyanins, including cyanidin derivatives, were shown to accumulate in cells, rendering them more resistant to irradiation with UV-B light and to reactive oxygen species (*1*, *36*, *39*). In addition, cyanidin monoglucosides such as cyanidin-glucoside and cyanidin-galactoside were shown to suppress photooxidation in retinal pigment epithelial cells at least in part by quenching singlet oxygen (*39*). These reports suggest that cyanidin derivatives may function as antioxidants to protect animal and human cells from oxidative damage.

A few works have suggested that the protective role of cyanidine glucosides may result from its ability to protect membrane integrity, in agreement with our results showing C3R protection of the lysosomal membrane in fibroblasts from oxidative stress induced by H_2O_2. For instance, Gabrielska et al. (*40*) have shown that cyanidin-3-glycosides and delphinidin-3-rutinoside have antioxidant activity in a liposomal membrane system, suggesting that cyanidin and cyanidin glycoside pigments may play a role as dietary antioxidants in the prevention of lipid peroxidation of cell membranes induced by reactive oxygen species in living systems. Earlier, Tsuda and co-workers have shown that cyanidin (Cy) and cyanidin-3-glucoside (C3G) are effective antioxidants in assays employing liposomes and rabbit erythrocyte membrane systems (*41*). Kurz et al. (*42*) have shown the protective effect against lysosomal membrane damage of two potent iron chelators: the hydrophilic desferrioxamine (dfo) and the lipophilic salicylaldehyde isonicotinoyl hydrazone (sih); both provided protection against oxidant-mediated lysosomal rupture. Here, the ability of C3R to inhibit ROS formation in fibroblast (NIH-3T3) cells following induction by metal oxidation was demonstrated, in a dose-dependent fashion. It may be suggested that the abilities of C3R to act as a ROS scavenger, as a scavenger of primary and late lipid peroxidation products and as a chelator of Fe^{2+} may all contribute to an overall protection of lysosomal membrane permeability from rupture, and avoid early apoptotic process in normal cells.

The results presented here show that C3R, the major anthocyanin in fresh fig fruits, may inhibit deleterious oxidation in several modes of action. This is supported by our results showing in vitro activities that are independent where C3R inhibits different oxidizing agents and may include ROS scavenging, Fe^{2+} chelation, and inhibition of lipid peroxidation; all may act individually or together to inhibit oxidative damage to membranes or cells, thus preventing early apoptotic processes. These results and the proven absorbance of C3R and other anthocyanins through the intestine (*1*, *41*) suggest a promising potential for C3R in preventing oxidative damage in biological systems and in treating diseases that involve free radical formation and oxidative stress.

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