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ARTICLE

EPR Studies of $O_2^{\bullet-}$, OH, and $^1O_2$ Scavenging and Prevention of Glutathione Depletion in Fibroblast Cells by Cyanidin-3-rhamnoglucoside Isolated from Fig (*Ficus carica* L.) Fruits

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Cyanidin-3-rhamnoglucoside (C3R) is the major anthocyanin in fresh fig fruits. In this study, the free radical scavenging potential of C3R was evaluated in vitro using several free radical generators. This naturally occurring anthocyanin was superior to other tested natural antioxidants in scavenging $ABTS^{\bullet+}$. Electron paramagnetic resonance served to determine the scavenging properties of C3R toward superoxide radical anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), and singlet radical ($^1O_2$). The protection of NIH-3T3 fibroblast cells was then tested as the inhibition of reactive oxygen species (ROS) formation in a dose-dependent manner. It was further demonstrated that treatment with C3R elevates the reduced glutathione (GSH) concentration and the redox ratio (GSH/GSSG) in fibroblast cells in a dose-dependent manner. Moreover, C3R reduced the induction of ROS by butathionine sulfoximine (BSO) and elevated the redox ratio. Thus, it is suggested that C3R in fresh fig fruits is a potent scavenger and may influence endogenous antioxidant systems of consumers.

KEYWORDS: Fig fruits; cyanidin-3-rhamnoglucoside (C3R); reactive oxygen species (ROS); NIH-3T3 fibroblast cells; butathionine sulfoximine (BSO); reduced glutathione (GSH); oxidative glutathione (GSSG); redox ratio (GSH/GSSG)

INTRODUCTION

Fruits and vegetables that contain high concentrations of phytochemicals have attracted considerable interest in recent years due to their potential health-promoting effects (*1*). These effects are commonly attributed to their antioxidant capacity (*2*). Epidemiological studies demonstrating the potential health benefits of consuming natural antioxidants (*3*) and advances in understanding the mechanisms of action of antioxidants in cells, tissue cultures, and animal models (*4*), as well as in humans (*5*), established the nutritional value of antioxidants from plants. Eberhardt and others suggested that the major antioxidant activity in fruits and vegetables is due to the presence of polyphenol and flavonoid compounds (*6*). Several flavonoids were shown to be better scavengers of peroxy radicals than vitamin E, vitamin C, or glutathione, which are produced by normal metabolism and play a major role in cellular defense against oxidative damage (*7*).

The fig fruit (*Ficus carica* L.) has been a typical component in the health-promoting Mediterranean diet for millennia. Today, the fig is an important crop worldwide for dry and fresh consumption (*8*, *9*). Recently, we described for the first time the isolation and chemical identification of cyanidin-3-rhamnoglucoside (cyanidin-3-rutinoside; C3R) (**Figure 1**), the main anthocyanin in the skin fig fruits, accounting for 95% of the total anthocyanin content. This natural compound is also distributed in several fruits and vegetables, such as fresh olives, mulberries, cherries, acai palm berries (*Euterpe oleracea* Mart), and black currant (*Ribes nigrum* L.) (*10*−*14*). We previously reported that antioxidant capacity measured by Trolox equivalent antioxidant capacity (TEAC) assay is tightly correlated with the anthocyanin fraction ($R^2 = 0.992$). Moreover, in the dark-colored fig variety (Mission), C3R contributes 33% of the total antioxidant capacity (*15*).

The present study was designed to characterize the antioxidant qualities of C3R purified from fig extract by C_{18} Sep-Pak cartridge and RP-LC as described before (*15*). Within this framework, we investigated the antioxidant capabilities of C3R in vitro compared to those of well-known antioxidants such as *trans*-resveratrol and ascorbic acid. In addition, we determined the direct action of C3R on reactive oxygen species such as singlet oxygen ($^1O_2$), superoxide radical anion ($O_2^{\bullet-}$), and hydroxyl radical ($^{\bullet}OH$). Other antioxidant capabilities, such as the ability

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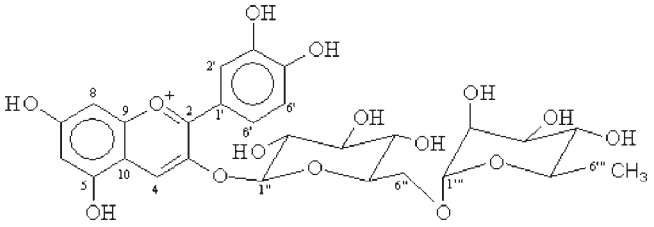

Figure 1. Chemical structure of cyanidin-3-rhamnoglucoside (C3R), the main anthocyanin in fig fruit (*Ficus carica* L.).

to scavenge reactive oxygen species (ROS) in an NIH-3T3 fibroblast cell line, were also determined. Finally, the influence of C3R on glutathione, the most important endogenous antioxidant in cells, was investigated.

MATERIALS AND METHODS

Materials. Trolox was obtained from Acros Organics, Morris Plains, NJ. Xanthine and xanthine oxidase, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), and 2,2,6,6-tetramethyl-4-piperidone (TEMP) were purchased from Sigma Chemicals (St. Louis, St. Louis, MO). 2′,7′-Dichloroflorofluorescin diacetate (DCFH-DA), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase, 2-vinylpyridine, $ABTS^{\bullet+}$ (2,2′-azinobis(3-ethylbenzothiazolone-6-sulfonate)), butathionine sulfoximine (BSO), bovine serum albumin (BSA), and (2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) were purchased from Sigma Chemicals (Rehovot, Israel). A 4×10^{-2} M stock solution of L(R:S) BSO was made fresh daily, dissolved in double-distilled water (DDW), and filtered using 0.22 μm Millipore filter. The solution was kept in subdued light at room temperature until used. NIH-3T3 fibroblast cells were obtained from the American Type Culture Collection (Manassas,VA).

Isolation of Cyanidin-3-rhamnoglucoside from Fresh Mission Fruits. Isolation and purification of C3R from fresh skin of Mission variety (dark fig fruits) were described by us in our first paper (*15*).

Antioxidant Activity in Cell-free Systems: Determination of Trolox Equivalent Antioxidant Capacity of C3R. The TEAC was measured by the $ABTS^{\bullet+}$ decolorization assay, using preformed $ABTS^{\bullet+}$ radical cation, and calculated relative to Trolox as described previously (*16*). All determinations were performed in triplicates.

Electron Resonance Spectroscopy (EPR) Measurements. *EPR Spectra*. The EPR spectra were recorded on a Bruker ER 100d X-band spectrophotometer (Bruker BioSpin Group, Billerica, MA). The measurements were repeated three times for each sample. After acquisition, the spectra were produced by Bruker WIN-EPR software version 2.11 for baseline correction, noise filtration, and integrating the signals. In all figures, the intensity is expressed in arbitrary units.

Xanthine−Xanthine Oxidase System. The reaction of xanthine and xanthine oxidase was used as a source of the superoxide radical anion, $O_2^{\bullet-}$ (*17*). Prior to performing EPR studies, we examined whether C3R inhibits xanthine oxidase activity by monitoring uric acid formation at 290 nm. The controls were measured by the same concentrations of C3R that were used in the EPR assay. There was no significant change in uric acid formation, indicating that C3R does not inhibit the enzyme. The typical reaction mixture consisted of DEPMPO (100 mM), xanthine (20 μM), xanthine oxidase (0.015 U), and DTPA (0.2 mM), in air-saturated PBS, pH 7.4. The assay mixture was transferred to a quartz capillary and fitted into the cavity of the Varian EPR spectrometer. DEPMPO represents an improvement over the widely used DMPO in terms of higher stability of the spin adducts (*18*).

Determination of Hydroxyl Free Radicals. DEPMPO served as a spin trap, and Fenton reaction was utilized to test the scavenging of $^{\bullet}OH$ by C3R (*19*). The reaction mixture contained 30 mM DEPMPO, 100 μM $FeSO_4$, and 10 mM H_2O_2, in the absence and presence of C3R, at a final volume of 100 μL and at final pH of 7.4. The widely used antioxidant propyl gallate was assayed in this system as a positive control. EPR was measured as follows: microwave frequency, 9.66 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; modulation amplitude, 0.6 G; time constant, 0.328 s; seep time, 336 s; and receiver gain, 2×10^5.

Photodetermination of Singlet Oxygen. Singlet oxygen was generated in the photoradiation riboflavin system. The reaction mixture contained 0.3 mM riboflavin, 30 mM 2,2,6,6-tetramethyl-4-piperidone (TEMP), and C3R. The mixture was irradiated by a visible light projector with a 25 W lamp at room temperature for 5 min, and EPR spectra were then recorded. The singlet oxygen detection was based on the specific reaction between $^1O_2$ and TEMP, which forms a stable and EPR detectable 4-O-TEMPO radical. The conditions of EPR measurement were as follows: microwave frequency, 9.66 GHz; modulation frequency, 100 kHz; microwave power, 15 mW; modulation amplitude, 1.0 G; time constant, 0.655 s; seep time, 336 s; and receiver gain 2×10^5.

Assays in Cell Culture. *Oxidative Stress in NIH-3T3 Fibroblast Cells*. NIH-3T3 fibroblast cells at a density of 4×10^4 viable cells/well were suspended in complete Dulbecco's modified Eagle's medium (DMEM) + 10% FCS and 1% penicillin−streptomycin solution. After 24 h, cells were washed twice in PBS (50 mM, pH 7.5) and exposed (or not) to 1.6 mM AAPH and 5.0 μM DCFH-DA for 20 min at 37 °C. The cells were rinsed twice with PBS and photographed by fluorescence microscopy. In other experiments, 3T3 cells were incubated with 20 μM C3R for 10 min at 37 °C. Thereafter, the cells were treated with 1.6 mM AAPH and 5.0 μM DCFH-DA for 20 min at 37 °C and then photographed by fluorescence microscopy.

Influence of BSO Treatment on Reactive Oxygen Species (ROS) Production in NIH-3T3 Fibroblast Cells. The concentration of ROS in the cells was assayed according to the method of Rosenkranz et al. with some modifications (*20*). NIH-3T3 fibroblast cells at a density of 1×10^4 viable cells/well were plated in 96-well microtiter tissue culture plates (Grenier Bio One GmbH, Kremsmünster, Germany) for 24 h, suspended in DMEM + 10% FCS and 1% penicillin−streptomycin solution. After 24 h, cells were exposed to 10 μM L(R:S) BSO with or without elevated concentrations of cyanidin-3-rhamnoglucoside (0−28 μM). After 30 min, cells were incubated with 5.0 μM 2′,7′-dichlorofluoescein-diacetate and then read with a Tecan fluorometer (wavelength 485/530 nm) (Tecan, Männedorf, Switzerland). The amount of intracellular ROS production was proportional to the green fluorescence.

Influence of BSO Treatment on Total Glutathione, GSH, and GSH to GSSG Ratio in NIH-3T3 Fibroblast Cells. The levels of total and GSSG were determined with the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) glutathione reductase assay according to the method of Axelsson et al. (*21*). Sample values were calculated from standard curves and expressed as nanomoles of GSH or GSSG per milligram of protein. NIH-3T3 fibroblast cells at a density of 1×10^4 viable cells/well were plated in 96-well microtiter tissue culture plates (Grenier Bio One GmbH) for 24 h. After 24 h, cells were washed twice in PBS (50 mM, pH 7.5) and exposed to 10 μM BSO and 0−40 μM C3R for 15 min. Total glutathione, GSH, and GSSG concentrations were analyzed using the same method as described above (*21*).

Influence of C3R on Total Glutathione, GSH, and GSSG in NIH-3T3 Fibroblast Cells. NIH-3T3 fibroblast cells were suspended in complete medium (DMEM) for 24 h. Cells were washed twice in PBS (50 mM, pH 7.5) and exposed to 0−40 μM C3R for 15 min. Total glutathione, GSH, and GSSG concentrations were analyzed using the same method as described above (*21*).

Cell Lysates and Protein Analysis. NIH-3T3 fibroblast cells that were grown and treated with C3R were washed twice with cold PBS (50 mM, pH 7.0) and transferred into 1 mL Eppendorf tubes. Cell lysates were prepared by freezing in liquid nitrogen and then heating for 5 min in a boiling water bath. The suspended cells were centrifuged at 10000 rpm for 10 min at 4 °C to pellet the cell debris. Protein concentration was measured according to the method of Bradford (*22*), with BSA as a standard.

Statistical Analysis. All determinations were based on at least three independent assays. Each test was measured in triplicates. 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

Antioxidant Activity of C3R in Cell-free System. *TEAC*. As a first measure of the antioxidant potential of the isolated anthocyanin C3R, its TEAC was determined by its ability to scavenge

Table 1. Relative Trolox Equivalent Antioxidant Capacity (TEAC) of Some Natural Antioxidants

antioxidant	IC_{50}[a] (μM)
cyanidin-3-rhamnoglucoside (C3R)	2.1 a
resveratrol	8.4 b
caffeic acid	13.0 c
ferulic acid	11.7 cd
ascorbic acid	14.3 d
Trolox	21.9 f

[a] Different letters indicate significant differences ($P < 0.05$).

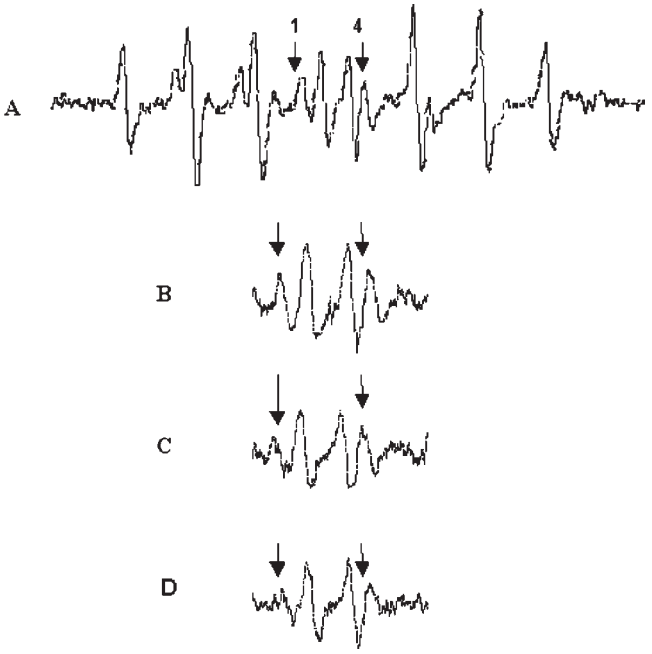

Figure 2. Influence of C3R on superoxide radical anion formation: (**A**) total spectrum (peaks 1 and 4 are the relevant peaks for superoxide radical anion); (**B**) control, 0 μM C3R; (**C**) 3.5 μM C3R (54% inhibition); (**D**) 15 μM C3R (70% inhibition). IC_{50} = 3.2 μM C3R.

the cation radical $ABTS^{\bullet+}$ and compared to that of Trolox (a water-soluble derivative of vitamin E) and to those of other antioxidants of plant origin (caffeic, ferulic, ascorbic acids, and resveratrol). These antioxidants differ from each other in their functional groups. The oxidative decolorization of the $ABTS^{\bullet+}$ cation was monitored, and the results expressed as IC_{50} showed C3R to be superior to other antioxidants (**Table 1**).

Effect of C3R on Superoxide Radical Anion ($O_2^{\bullet-}$). Superoxide radical anion is generated from triplet oxygen by a reduction reaction. The reactivity of $O_2^{\bullet-}$ is not very much greater, but its toxicity is due to its ability to form $^{\bullet}OH$, the most destructive species, and from its ability to recycle Fe^{2+} from Fe^{3+}.

Superoxide radical anion is produced when xanthine oxidase acts on xanthine in the presence of oxygen (*17*). The ability of C3R to scavenge $O_2^{\bullet-}$ was monitored. The spectrum of DEPMPO-OOH generated in the xanthine−xanthine oxidase system is presented in **Figure 2A**,**B**, which show typical EPR spectra resulting from the interactions of an uncoupled electron with a primary atom, as well as with the secondary β and γ protons. At 3.5 μM cyanidin-3-rhamnoglucoside, about 54% inhibition in the intensity of the EPR signal was obtained (**Figure 2C**). At 15.0 μM, about 70% inhibition in the intensity of the EPR signal was observed (**Figure 2D**). The IC_{50} of C3R toward superoxide radical anion was calculated to be 3.2 μM.

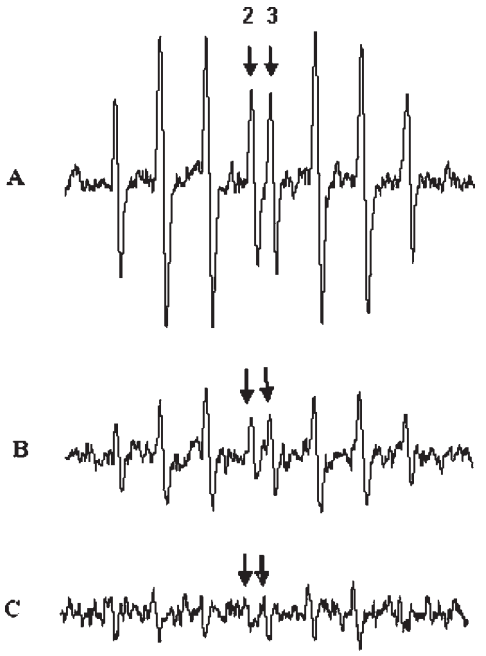

Figure 3. Influence of C3R on hydroxyl radical formation (peaks 2 and 3 are the relevant peaks for hydroxyl radical): (**A**) 0 μM C3R; (**B**) 38.0 μM C3R (61% inhibition); (**C**) 76.0 μM C3R (74% inhibition). IC_{50} = 23 μM C3R.

Scavenging of Hydroxyl Radical ($^{\bullet}OH$) *by C3R*. The ability of C3R to scavenge hydroxyl radical was measured. Using the Fenton reaction, a well-known $^{\bullet}OH$ generator, and the spin trap DEPMPO, a typical EPR spectrum of DEPMPO was obtained (**Figure 3**). In the presence of increasing concentrations of C3R, the concentration of DEPMPO−OH adduct decreased by up to 77%. The IC_{50} of C3R toward hydroxyl radical was calculated to be 23.0 ± 3.1 μM. In the same assay, propyl gallate exhibited a similar scavenging potential toward the hydroxyl radical (21 ± 2.6 μM). There was no significant difference between the results ($P < 0.05$).

Scavenging of Singlet Oxygen ($^1O_2$) *by C3R*. Singlet oxygen is generated by inverting the spin of one of the electrons of the two outer orbitals, removing the quantum-mechanical spin restrictions of molecular oxygen (*23*). A typical EPR spectra of the spin adduct of singlet oxygen is demonstrated in **Figure 4**. In the presence of 0.2, 0.8, 1.8, and 3.8 μM C3R, the signal intensity decreased by 27, 54, 73, and 83%, respectively. The IC_{50} of C3R toward singlet oxygen was calculated to be 0.7 μM.

Antioxidant Activity in NIH-3T3 Fibroblast Cells. *Oxidative Stress in NIH-3T3 Fibroblast Cells Induced by AAPH*. The effect of C3R on oxidative stress in NIH-3T3 fibroblast cells, induced by the addition of AAPH, was investigated using DCFH-DA, an indicator sensitive to free radicals. When DCFH-DA is oxidized by ROS, it is converted to dichlorofluorescein (DCF) and emits green fluorescence (*24*). The fluorescence intensity is a function of the ROS concentration in the culture medium. The ability of C3R to eliminate ROS production and to reduce oxidative stress induced by AAPH in NIH-3T3 fibroblast cells was tested in this assay by fluorescence microscopy (**Figure 5**). **Figure 5A** shows the basal level of ROS in NIH-3T3 fibroblast cells. The production of low levels of ROS in cells is important to maintain their normal vitality (*25*). Oxidative stress induced by AAPH results in intense green fluorescence (**Figure 5B**), whereas adding 20 μM C3R to the incubation medium prior to exposure to AAPH inhibited oxidative response and reduced ROS generation (**Figure 5C**).

Oxidative Stress in NIH-3T3 Fibroblast Cells Induced by BSO Treatment. BSO, a known selective inhibitor of GSH synthesis, induces GSH depletion from cells (*26*). We analyzed and quantified the extent of the ROS formation in NIH-3T3 cells by measuring fluorescence from the DCF that was produced. Our results revealed that 10 μM C3R reduced approximately 40% of the total ROS content 15 min after BSO treatment. Reduction of the ROS formation was evident 1 h after BSO treatment, and after 24 h, the cells' morphology was altered (data not shown). Cotreatment with 10 μM BSO for 15 min with or without various concentrations of C3R (0−28 μM) reduced the induction of ROS in a dose-dependent manner, by up to 70% inhibition compared to control, respectively (**Figure 6**). These data demonstrate that cotreatment of 10 μM BSO together with C3R at concentrations of ≤9.4 μM reduced the negative influence of BSO by reducing the ROS concentration. The IC_{50} for C3R in this assay was determined to be 16.0 μM.

Influence of BSO Treatment on Total Glutathione, GSH, and GSH/GSSG in NIH-3T3 Fibroblast Cell. The cellular content of total glutathione (GSH + GSSG), as well as its redox change, is recognized as a sensitive indicator of oxidative stress. We analyzed the change of glutathione status in NIH-3T3 cells in response to BSO. Data demonstrating the influence of BSO treatment on total glutathione, reduced glutathione (GSH), and the ratio of GSH to GSSG (GSH/GSSG) in NIH-3T3 fibroblast cells are given in **Table 2**. Our results revealed an approximately 45% reduction in total glutathione content, a 48% reduction in GSH content, and an increase of 27% in GSSG levels 15 min after BSO treatment. At the same time, the relative ratio of GSH to GSSG (GSH/GSSG), which is known as a redox ratio (*24*), was reduced by 60.5%.

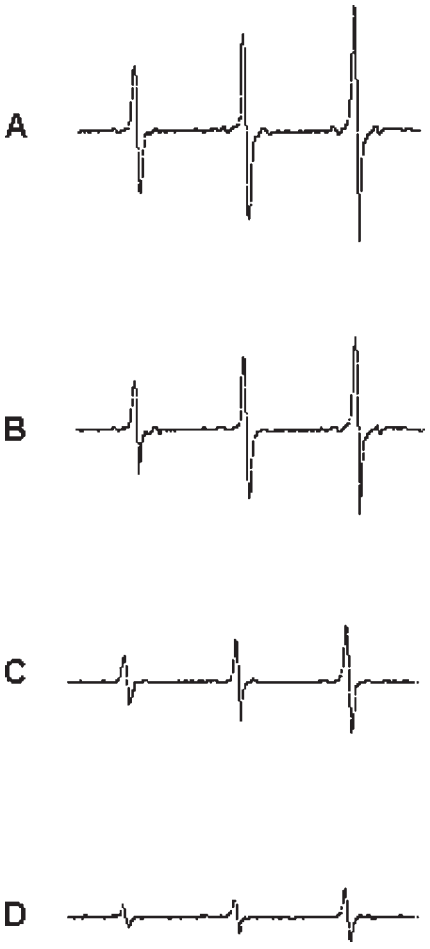

Figure 4. Effect of cyanidin-3-rhamnoglucoside on singlet oxygen ($^1O_2$) formation: (**A**) control, 0 μM C3R; (**B**) 0.2 μM C3R (27% inhibition); (**C**) 0.8 μM C3R (54% inhibition); (**D**) 3.8 μM C3R (83% inhibition). All three peaks are relevant for singlet oxygen. IC_{50} = 0.7 μM C3R.

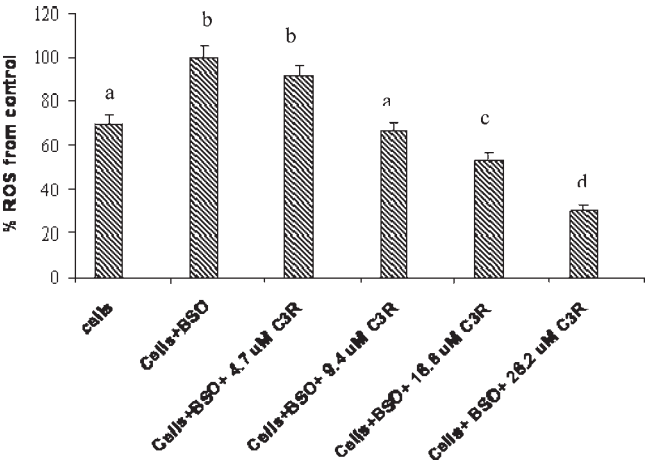

Figure 6. Relationship between ROS inhibition in NIH-3T3 fibroblast cells and C3R concentration. Cells were exposed to 10 μM L(R:S) BSO (a γ-glutamylcysteine synthase inhibitor) with or without elevated concentrations of cyanidin-3-rhamnoglucoside (0−28 μM) for 30 min. After 30 min, cells were incubated with 5.0 μM 2′,7′-dichlorofluoescein diacetate and then read with a Tecan fluorometer (wavelength 485/530 nm). The amount of intracellular ROS production was proportional to the green fluorescence. Cotreatment of 10 μM BSO together with C3R at concentrations of ≤9.4 μM reduced the negative influence of BSO by reducing the ROS concentration. IC_{50} = 16 μM of C3R. Different letters indicate significant differences ($P < 0.05$).

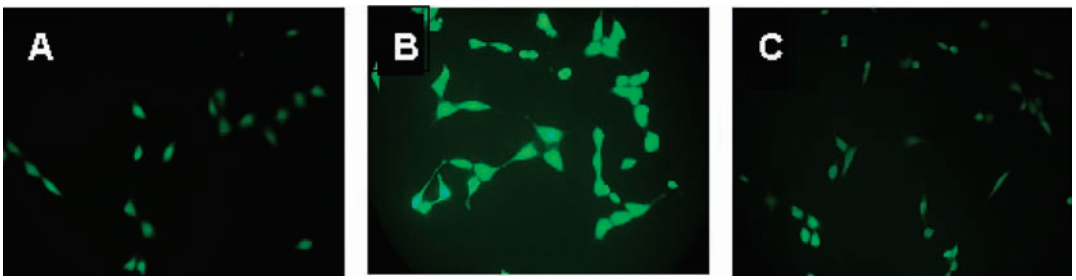

Figure 5. Effect of C3R on oxidative stress induced by AAPH in NIH-3T3 fibroblast cells: (**A**) control (the basal amount of ROS in NIH-3T3 fibroblast cells); (**B**) NIH-3T3 fibroblast cells exposed to AAPH at a final concentration of 1.6 mM; (**C**) NIH-3T3 fibroblast cells treated with 20 μM of C3R prior to exposure to 1.6 mM AAPH. Oxidative stress was measured by fluorescence microscopy using DCFH-DA. When DCFH-DA was oxidized by reactive oxygen species (ROS), it was converted to DCF and emitted green fluorescence. The fluorescence intensity is related to the amount of ROS concentration in the cells.

Table 2. Influence of Cyanidin-3-rhamnoglucoside (C3R) and BSO Levels on Glutathione Parameters[a]

C3R concn (μM)	BSO concn (μM)	total glutathione	GSH (nmol/mg of protein)	GSSG (nmol/mg of protein)	GSH/GSSG
0	0	14.3 ± 0.8 a	13.6 ± 0.9 a	0.32 ± 0.01a	42.5
0	10	7.9 ± 0.9 b	7.1 ± 1.0 b	0.44 ± 0.02 b	16.8
5	10	11.4 ± 0.6 c	10.7 ± 0.7 c	0.35 ± 0.02 c	30.7
10	10	12.1 ± 0.7 c	11.5 ± 0.8 c	0.30 ± 0.02 a	38.2
20	10	14.0 ± 0.7 a	13.3 ± 0.6 a	0.30 ± 0.02 a	44.3
40	10	14.2 ± 1.3 a	13.8 ± 1.2 a	0.20 ± 0.03 d	62.6

[a] Different letters in the same column indicate significant differences ($P < 0.05$). Data are expressed as means ± standard deviations ($n = 4$).

Cotreatment with 10 μM BSO and various concentrations of C3R from 0 to 40 μM elevated the total glutathione and reduced the GSSG levels compared to 10 μM BSO. Exposure to 10 μM BSO and 5 μM C3R elevated the total glutathione and GSH levels and the redox ratio (GSH/GSSG) by approximately 30%; the redox ratio (GSH/GSSG) was elevated by 45% compared to 10 μM BSO. Exposure to 10 μM BSO and 10 μM C3R elevated the total glutathione and GSH levels and the redox ratio by 35, 38, and 56%, respectively. Cotreatment with 10 μM BSO and 10 μM C3R elevated the total glutathione and GSH levels and the redox ratio by 44, 47, and 62%, respectively. In addition, exposure to 10 μM BSO and 40 μM C3R elevated the total glutathione and GSH levels and the redox ratio by 44, 49, and 73%, respectively. There were no significant differences between cells exposed to 20 or 40 μM C3R in total glutathione and GSH levels, however, there were significant differences between cells exposed to 20 or 40 μM C3R in GSSG levels and GSH/GSSG ratios (**Table 2**). From these data (**Table 2**), we can deduce that 20−40 μM C3R may restore the normal glutathione redox balance in NIH-3T3 fibroblast cells after oxidative stress induced by BSO treatment.

Effect of C3R on Total Glutathione, GSH, and GSSG in NIH-3T3 Fibroblast Cells. GSH (L-γ-glutamyl-L-cysteinyl-glycine) is an important endogenous antioxidant (*27*). The influence of C3R on the glutathione system was measured in NIH-3T3 cells without inducing any oxidative stress. When cells were treated with increasing levels (5−40 μM) of C3R, total glutathione and GSH concentrations were elevated compared to baseline (0 μM C3R). In addition, there were no significant differences in the GSSG concentration among all of the treatments (**Figure 7**).

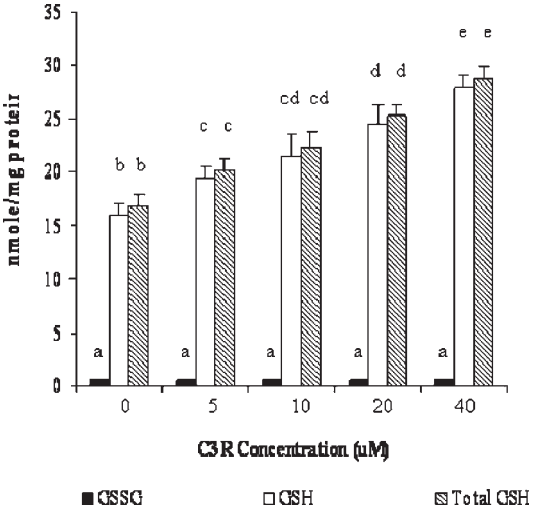

Figure 7. Influence of different concentrations (0−40 μM) of cyanidin-3-rhamnoglucoside (C3R) on total glutathione, GSH, and GSSG levels in NIH-3T3 fibroblast cells. Different letters indicate significant differences ($P < 0.05$).

DISCUSSION

The antioxidant capacity of fresh fig fruit and the potential health benefits of its consumption have not been described in detail. In a previous work, we have shown C3R (**Figure 1**) to be its main anthocyanin, contributing 36% of the total antioxidant capacity of dark purple fruits, such as the Mission variety (*15*). Here, several assays were used to study the antioxidant capacity of C3R in vitro and in NIH-3T3 fibroblast cells in an effort to characterize its antioxidant properties. The TEAC assay was performed to estimate the relative antioxidant capacity of C3R compared to others natural antioxidants such as *trans*-resveratrol, caffeic acid, ferulic acid, vitamin C, and Trolox (**Table 1**). These antioxidants differ from each other in their functional groups. It is clear from data obtained that the natural antioxidant isolated from dark purple fig skins (Mission variety) is very efficient compared to other known antioxidants. This is in agreement with other studies suggesting that among all common fruits and vegetables in the diet, berries, especially those with dark blue or red colors, have the highest antioxidant capacity (*28*). Using EPR spectroscopy, we showed here that C3R has free radical and singlet oxygen scavenger activity. The ability of C3R to inhibit ROS generation was related to its ability to scavenge superoxide radical anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) (**Figures 2−4**). Scavenging of superoxide radical anion is particularly important, because this radical is ubiquitous in aerobic cells, and despite its mild reactivity, it is a potential precursor of the aggressive hydroxyl radical in the Fenton and Haber−Weiss reactions (*29*). This broad spectrum of antioxidant activity is not characteristic of all natural antioxidants that have been studied (*30*). Boveris et al. (*31*) have shown that *Ginkgo biloba* extract was able to inhibit $O_2^{\bullet -}$ and $^{\bullet}OH$ generation, but wheat and alfalfa extracts were not able to generate these reactive oxygen species. Curcumin exhibited potent quenching effect on singlet oxygen but was found to be an ineffective superoxide and hydroxyl radical scavenger (*32*). *trans*-Resveratrol was found to act as a superoxide radical anion scavenger. In addition, chunganenol, a resveratrol hexamer, exhibited potent and selective quenching effects on singlet oxygen (*33*, *34*).

4,5-*O*-Trigalloylquinic acid isolated from *Pistacia lentiscus* L. leaves was reported as an effective derivative against $^{\bullet}OH$ and $O_2^{\bullet -}$ free radicals compared to the monomer and dimer of this compound (*35*). The IC_{50} for $^{\bullet}OH$ radicals was 26.6 ± 2.9 μM, similar to our results (23.0 ± 3.1 μM). The IC_{50} values for $O_2^{\bullet -}$ were 16.8 ± 2.4 μM for 4,5-*O*-trigalloylquinic acid and 3.2 ± 0.24 μM for C3R (*35*). Propyl gallate, a widely used antioxidant, exhibits scavenging activity toward the hydroxyl radical (*30*). The hydroxyl radical scavenging qualities toward $^{\bullet}OH$ were also measured in our study, showing an IC_{50} of 21.0 ± 2.6 μM. Our results showed that compared with 4,5-*O*-trigalloylquinic acid, C3R is a better $O_2^{\bullet -}$ scavenger and exhibits similar scavenging potential toward $^{\bullet}OH$ compared to galloylquinic acid and propyl gallate. In addition, Cucurbitacin glucosides (B + E) were found to inhibit hydroxyl radical, superoxide radical anion, and singlet

oxygen. IC_{50} values were 0.38, 8, and 11 mM, respectively. These values are higher compared to those for C3R (23.0, 3.2, and 0.7 μM, respectively) (*36*).

Structural features have been shown to play a major role in the antioxidant and scavenging properties of plant-derived antioxidants. C3R has a catechol group on its B ring, which has a low redox potential ($0.23 < E_7 < 0.75$ V). Because of that, C3R is thermodynamically able to reduce highly oxidizing free radicals with redox potentials in the range of 2.13−1.0 V, such as superoxide, hydroxyl, peroxyl, and alkoxyl radicals by hydrogen atom donation. In addition, the catechol group lends high stability to the aroxyl radical via hydrogen bonding or by expanded electron delocalization. The aroxyl radical may react with a second radical, acquiring a stable structure (*29*).

In this study, the antioxidant properties of C3R were examined in cell culture as well. C3R inhibited ROS formation in NIH-3T3 fibroblast cells (**Figure 5**). The incorporation of anthocyanins both in the plasma membranes and in the cytosol (*37*) may explain the activity of C3R against ROS in cultured cells.

Cells contain endogenous antioxidant defense mechanisms to scavenge ROS and maintain the redox balance. GSH and glutathione peroxidase (GSH-Px) are important endogenous antioxidants, and the GSSG/GSH ratio is regarded as a sensitive indicator of oxidative stress (*38*, *39*). Depletion of GSH by using the GSH synthetase inhibitor, BSO, disturbs the redox balance and enhances the vulnerability of cells to oxidative stress (*40*, *41*). In this study, the critical role of GSH as an antioxidant in NIH-3T3 cells was demonstrated by showing that 10 μM BSO caused an increase in ROS levels (**Figure 6**). Cotreatment of 10 μM BSO together with C3R at concentrations of ≤9.4 μM reduced the negative influence of BSO by reducing the ROS concentration (**Figure 6**).

On the basis of our measurements, 10 μM BSO reduced the redox ratio (GSH/GSSG) in NIH-3T3 fibroblast cells by reducing the GSH levels and elevating the GSSG concentrations. Increasing levels of C3R (from 5 to 40 μM) reduced the negative influence of BSO by enhancing the redox ratio (**Table 2**). In addition, 20−40 μM C3R may restore the normal glutathione redox balance in NIH-3T3 fibroblast cells after oxidative stress induced by BSO treatment. Cereser and co-workers (*42*) showed that under normal growth conditions, control NIH-3T3 fibroblast cells exhibit a concentration of GSH similar to that we have shown (**Table 2**). In addition, induction of fibroblasts with thiram (dithiocarbamate fungicide) results in rapid depletion of GSH within 15 min. These results are in agreement with our results.

In NIH-3T3 fibroblast cells, C3R itself was found to elevate total glutathione levels and redox ratio in a concentration-dependent manner by increasing GSH levels (**Figure 7**). Shih and co-workers (*43*) reported that anthocyanins, especially cyanidin, activate expression of glutathione-related enzymes (glutathione reductase, peroxidase, and *S*-transferase) and prevent intracellular glutathione (GSH) depletion in the rat hepatocyte clone 9 cell line.

Researchers have focused on antioxidant administration diminishing oxidative stress in vitro (*44*). Curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), gossypin (pentahydroxy glucosyl flavone), and mangiferin (1,3,6,7-tetrahydroxyxanthone-C2-β-D-glucoside) are polyphenol compounds that differ from each other by their functional groups (*44*−*46*). They have been shown to inhibit ROS generation in several cell lines through quenching of reactive oxygen intermediates and by increasing the GSH/GSSG ratio (*44*−*46*). Studies showed that the increase in GSH level is caused by activation of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in glutathione biosynthesis (*46*, *47*), or by modulation of the expression of glutathione-related enzymes such as GPx (*48*). Borras and co-workers (*49*) have shown that GSH regulates telomerase activity in NIH-3T3 cells. Telomerase activity has been associated either with cancer, when activity is increased, or with cell cycle arrest, when it is decreased. Depletion of GSH by BSO reduces telomerase activity and delays cell growth.

Anthocyanin metabolism is still not fully understood, and information in the scientific literature is controversial. There is evidence that cyanidin-3-glycosides are metabolized as glucuronidated and methylated versions of the parent compounds, which affect absorption and molecule redox (*50*). On the other hand, Uhley and co-workers (*51*) showed that the consumption of whole fruit tart cherries by healthy humans resulted in the appearance of two unmodified anthocyanins in plasma: cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside. In another study, researchers suggested that 3-*O*-β-rutinosyl anthocyanins, including C3R, were directly absorbed and distributed to the blood (*52*).

In this study, we have shown that C3R, the major anthocyanin in fresh fig fruits, was superior to other tested natural antioxidants in scavenging $ABTS^{\bullet+}$. In addition, by EPR C3R was noted as a sufficient scavenger toward $O_2^{\bullet-}$, $^{\bullet}OH$, and $^1O_2$. The inhibition of ROS formation in NIH-3T3 fibroblast cells in a dose-dependent manner was also tested. We further demonstrated that treatment with C3R elevates the GSH concentration and the redox ratio in NIH-3T3 fibroblast cells in a dose-dependent manner. Moreover, C3R reduced the induction of ROS by BSO and elevated the redox ratio. Thus, our results suggest that the antioxidant capacities of C3R, the major anthocyanin in fresh fig fruits, may contribute to their health benefits.

Future studies should determine the abilities of C3R on endogenous antoxidant enzymes such as superoxide dismutases (SOD), GSH-Px, catalase (CAT), and γ-GCS, the rate-limiting enzyme in glutathione biosynthesis. It will also be of interest to determine whether treatment with C3R can reduce the side effects of chemotherapeutic drugs such Adriamycin that generate free radicals, reduce GSH levels, and cause apoptosis of normal cells in the brain and heart (*53*).

Note Added after ASAP Publication

This paper was published on the Web on May 5, 2010, with an error to Figures 1 and 3. The corrected version was reposted on May 25, 2010.

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