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Assessment of free radicals scavenging activity of seven natural pigments and protective effects in AAPH-challenged chicken erythrocytes

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ABSTRACT

The purpose of this study was to compare the antioxidant capacities of seven natural pigments including the fat-soluble pigments curcumin, lycopene, lutein and β-carotene and water-soluble pigments – betalain, capsanthin and cyanidin-3-rutinoside relative to a commonly-used synthetic food antioxidant BHA. The antioxidant capacities of seven pigments and BHA were evaluated based on their ability to quench several free radicals, including DPPH, ABTS, $O_2^{\cdot-}$, H_2O_2 as well as using FRAP assay. Specifically, curcumin and cyanidin-3-rutinoside, which showed the highest antioxidant capacities, were further investigated using a chicken erythrocyte model. After separating pretreatments of the two pigments, AAPH was added to the erythrocyte-pigment medium to induce oxidative stress. Then the attenuation effects of the two pigments on AAPH-induced oxidative damage in chicken erythrocytes were assessed. It was found that both curcumin and cyanidin-3-rutinoside significantly attenuated apoptosis and hemolysis, decreased MDA content, and increased T-SOD activity in a time- and dose- dependent manner.

1. Introduction

Oxidative stress is an imbalance between the efficiency of the antioxidant defense system and the free radical generation system. Free radicals consist of radical oxygen species (ROS) and radical nitrogen species (RNS). ROS such as hydroxyl radical (**.**HO), superoxide anion radical ($O_2^{\cdot-}$), and hydrogen peroxide (H_2O_2) are highly reactive species and involved in many pathological conditions. They play a pathological role in early onset of oxidative stress and are capable of damaging biologically relevant molecules, such as DNA in the nucleus, and lipids in plasma membranes of cells (Lushchak, 2010; Nordberg & Arner, 2001). Cellular defense systems, including enzymatic and non-enzymatic antioxidants, protect the organisms against oxidative stress. In addition to endogenous antioxidant systems, frequent consumption of diets rich in natural antioxidants also exhibits increased resistance to oxidative stress and is associated with a lower risk of many oxidative stress-related diseases.

Edible pigments are compounds with various brilliant colors, which are consumed worldwide as adjuncts to enhance the food appearances of products. According to their origins, edible pigments are divided into natural- and synthetic-derived pigments. Natural pigments are mainly isolated from plants, microorganisms and animal tissues through physical extraction. In contrast, synthetic pigments are much more popular and make up the majority of supply in food markets due to their lower costs. However, with the development of food pigment research, public concerns about the safety of synthetic pigments used in foods has become a serious issue specifically regarding potential side effects and disease risks in humans. In recent years, natural pigments have merited more interest owing to their markedly fewer negative effects and greater health benefits, leading them to be a promising candidate for both health benefits and commercial needs.

Most of the edible natural pigments are either water- or lipid-soluble. Carotenoids, such as β-carotene, lycopene and lutein, are a major class of lipophilic colorants and free radical scavengers (Müller, Fröhlich, & Böhm, 2011). The red carotenoid, mainly consisting of capsanthin and capsorubin, is a popular food pigment and is also found in culinary spices (Stahl & Sies, 2003). Curcumin is a yellow hydrophobic pigment isolated from the rhizome (turmeric) of the herb *Curcuma longa*, which exerts various beneficial effects on human health. Recent scientific research has revealed that curcumin is a hot spot in animal husbandry, pharmaceutical and food processing industries (Ak & Gulcin, 2008). Anthocyanins, soluble compounds in aqueous medium, are capable of enhancing the appearances of food and preventing lipid peroxidation in different systems (Kähkönen & Heinonen, 2003). Betalains, composed of red–purple betacyanins and yellow betaxanthins, are also soluble in aqueous mediums (Delgado-Vargas, Jiménez, &

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Paredes-López, 2000). Strong evidence demonstrated that the afore-mentioned natural pigments could not only provide food with brilliant appearances, but also possess antioxidant capacities against oxidative stress (Delgado-Vargas et al., 2000).

Current literature is replete with descriptions of the individual antioxidative potencies of different natural pigments. However, to the best of our knowledge, limited information is available on the comparative antioxidant capacities across natural pigments. The first objective of this study was to systematically compare the *in vitro* antioxidant capacities of the seven selected natural pigments including the fat-soluble curcumin, lutein, lycopene, β-carotene, and the water-soluble cyanidin-3-rutinoside, betalain, and capsanthin. Butylated hydroxyanisole (BHA), commonly used synthetic antioxidant in food industry, was adopted as a reference radical scavenger in the first part. The second objective was to screen the protective effects of the natural pigments in a chicken erythrocyte model. Erythrocytes are susceptible to free radical induced oxidative damages and the membrane domain of erythrocytes are highly conserved among species, including the birds. The erythrocyte model is much closer to the physiological condition of living cells and their results are more accredited to be the foundation of the *in vivo* study. Therefore, we chose the chicken erythrocytes which contain the nuclear in contrast to the human ones. Moreover, based on the results of free radical scavenging assays, we found that curcumin and cyanidin-3-rutinoside showed the largest antioxidant capacities among these seven pigments. Thus, both of them were selected to study for the protective effects (e.g., hemolysis, apoptosis, lipid peroxidation and total superoxide dismutase activity) against 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) induced-oxidative damage in the chicken erythrocyte.

2. Materials and methods

2.1. Pigments

Curcumin, lutein, lycopene, β-carotene, cyanidin-3-rytinoside, betalain, and capsanthin were obtained from Sigma Chemical Co., Ltd.

2.2. Chemicals

2,2-Dipheny-L-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium chloride (NBT), 2,4,6-tri(2-pyridyl)-*S*-triazine (TPTZ), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), dimethylsulfoxide (DMSO), potassium persulfate ($K_2S_2O_8$), and butylated hydroxyanisole (BHA) were purchased from Sigma Chemical Co., Ltd. All other chemicals used in the present study were obtained from Shanghai Chemical Agents Company, China and were of analytical grade.

2.3. DPPH radical scavenging activity

DPPH radical scavenging activity was measured according to the method of (Liu, Shi, Colina Ibarra, Kakuda, & Jun Xue, 2008) with modifications. Briefly, DPPH was dissolved in ethanol to a 0.1 mM solution. Aliquots of DPPH solution and sample solutions at various concentrations (10–60 μg/ml) were mixed and shaken vigorously. Then the absorbance was determined at 517 nm after incubation for 30 min in the darkness at room temperature. The percent DPPH scavenging effect was calculated using the following equation:

$$\text{DPPH scavenging effect } (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%$$

where A_{control} was the absorbance of the control reaction and A_{sample} was the absorbance in the presence of pigments or reference substances.

2.4. ABTS radical cation decolorization assay

The spectrophotometric analysis of ABTS radical cation ($ABTS^{\cdot+}$) scavenging activity was determined according to the method of Re et al. (1999). 7 mM of $ABTS^{\cdot+}$ stock solution in water was prepared. $ABTS^{\cdot+}$ was produced by reacting ABTS stock solution with 2.45 mM $K_2S_2O_8$ and allowing the mixture to stand in the dark at room temperature for 12–16 h. Before usage, the $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.02 at 734 nm. The mixture of 1 ml of $ABTS^{\cdot+}$ stock solution and 3 ml of sample solutions at various concentrations (10–60 μg/ml) were incubated at 30 °C for 30 min. Then the absorbance was recorded at 534 nm. Reagent blank reading was taken as the control. The scavenging activity of $ABTS^{\cdot+}$ was calculated using the following equation:

$$\text{ABTS}^{\cdot+}\text{scavenging effect } (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%$$

where A_{control} was the absorbance of the $ABTS^{\cdot+}$ reaction and A_{sample} was the absorbance in the presence of pigments or reference substances.

2.5. Superoxide radical scavenging activity

Superoxide radical ($O_2^{\cdot-}$) scavenging activity was measured using the method of Chen and Yen (2007). Briefly, PMS, NADH and NBT were dissolved in phosphate buffer (0.1 M, pH = 7.4) to a 60, 468 and 150 μM solutions, respectively. The reaction mixture contained 1 ml of sample solutions at various concentrations (10–60 μg/ml), 1 ml of PMS solution, 1 ml of NADH solution and 1 ml of NBT solution, and were incubated at room temperature for 5 min. Then the absorbance was determined at 560 nm. The reaction was started when adding 1 ml of PMS solution to the mixture. Decrease in absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of $O_2^{\cdot-}$ generation was calculated using the following formula:

$$O_2^{\cdot-}\text{scavenging effect } (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%$$

where A_{control} is the absorbance of the control reaction and A_{sample} is the absorbance of the samples.

2.6. Hydrogen peroxide scavenging activity

The hydrogen peroxide (H_2O_2) scavenging assay was carried out following the procedure of Ak and Gulcin (2008) with some modifications. A solution of 43 mM H_2O_2 was prepared in phosphate buffer (0.1 M, pH = 7.4) at 20 °C. Then 3.4 ml of sample solutions at various concentrations (10–60 μg/ml) in phosphate buffer (0.1 M, pH = 7.4) was mixed with 0.6 ml of a hydrogen peroxide solution. Then the absorbance of the reaction mixture was recorded at 230 nm. The blank solution contained the phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging activities by both sample and standard compounds was calculated using the following equation:

$$H_2O_2\text{scavenging effect } (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%$$

where A_{control} is the absorbance of the control reaction (containing all reagents except the test sample) and A_{sample} is the absorbance of the test samples.

2.7. Ferric-reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power of thyme extracts was measured according to Benzie and Strain (1996) with some modifications. The FRAP reagent consist of 10 volumes of acetate buffer (0.3 M, pH = 3.6), 1 volume of TPTZ (10 mM) in hydrochloric acid (40 mM), and 1 volume of ferric chloride (20 mM). Just before the reaction, 1.5 ml of FRAP reagent, freshly prepared and warmed at 37 °C, was mixed with 150 μl of distilled water and 50 μl of sample solutions at various concentrations (10–60 μg/ml). The absorbance at 593 nm was determined after a 30 min incubation period in the dark at 37 °C. The reducing power was expressed as micromoles Fe(II) per gram fresh weight. A standard curve in the range 5–150 μM of $FeSO_4{\cdot}7H_2O$ was used for calibration.

2.8. Assay for hemolysis of chicken erythrocytes

Freshly collected chicken blood, obtained from a single genetic line, was mixed with heparin and centrifuged at 1500*g* for 10 min at 4 °C. After removal of plasma and buffy coat, erythrocytes were washed three times with cool PBS buffer (pH = 7.4, containing 150 mM NaCl, 1.9 mM Na_2HPO_4 and 8.1 mM NaH_2PO_4) and finally resuspended in the same PBS buffer.

In order to induce the free radical chain oxidation in erythrocytes, the aqueous peroxyl radicals were generated by thermal decomposition of AAPH (an azo compound) in oxygen. Briefly, a 2% suspension of chicken erythrocytes in PBS buffer were pre-incubated for 30 min at 37 °C with curcumin (0.5–10 μM) or cyanidin-3-rutinoside (0.5–10 μM), followed by incubation with or without AAPH (final concentration 75 mM) for 5 h with gentle shaking. Hemolysis of erythrocytes was determined spectrophotometrically as described previously by (Magalhaes et al., 2009) with minor modifications. Aliquots of the reaction mixture were taken out and centrifuged at each hour of the 5 h of incubation. The supernatant fraction was then discarded, and the pellet diluted with saline (1:7, v/v, 150 mM), and centrifuged at 1500*g* for 10 min. The percentage of hemolysis was determined by measuring the absorbance of the supernatant (A) at 540 nm and compared with that of complete hemolysis (B) by treating an aliquot with the same volume of the reaction mixture with distilled water. The hemolysis percentage was calculated using the formula: A/B × 100%. Three independent experiments were used for these calculations.

2.9. Measurement of erythrocyte lipid peroxidation and T-SOD activity

Lipid peroxidation was assessed indirectly through measurement of the MDA levels in erythrocytes. After repeated (three times) sonication and a single freeze–thaw procedure, MDA levels and T-SOD activity in erythrocytes were determined by analysis kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, People's Republic of China), according to the instructions of the manufacturer. Data for T-SOD was expressed as specific activity units per gram of hemoglobin (U/g Hb) and MDA was expressed in millimol per gram of hemoglobin (mmol/g Hb) in erythrocytes.

2.10. FACS analysis of annexin V binding

Phosphatidylserine exposure on the outer leaflet of the plasma membrane was measured using an Alexa Fluor® 488 annexin V/Dead cell apoptosis kit (Invitrogen, cat. no. V13245) according to the manufacturer's instructions. Briefly, following the preincubation of AAPH and pigments, the erythrocytes were washed twice with cool PBS buffer (pH = 7.4, containing 150 mM NaCl, 1.9 mM Na_2HPO_4 and 8.1 mM NaH_2PO_4) and resuspended (2% suspension) in 1× annexin-binding buffer. Then, the cell density was determined and diluted in 1× annexin-binding buffer to about 1×10^6 cells/ml. A sufficient volume of the above erythrocyte suspension was stained with Annexin V-FITC and propidium iodide (1:9 dilution) staining solution in dark for 15 min at room temperature. After incubation, the forward scatter (FSC) of erythrocytes was determined, and annexin V fluorescence intensity was measured in FL-1 with 488 nm excitation wavelength and 530 nm emission wavelength on a FACS caliber (BD, Heidelberg, Germany).

2.11. Statistical analysis

Data were analyzed using the SPSS 17.0 statistical package (SPSS, Inc., Chicago, IL, USA). Multiple comparisons between groups were performed by one-way ANOVA supplemented with Tukey's HSD post hoc test. Significance was accepted at p lower than 0.05.

3. Results and discussion

Natural pigments as antioxidants have attracted considerable attention because of their potentially beneficial effects on disease prevention in animals and humans. On the basis of previous studies (Reddy, Alexander-Lindo, & Nair, 2005), we selected seven natural pigments, both with excellent coloring property and free radicals quenching potency. Among these pigments, four are fat-soluble, and the rest are water-soluble. The focus of the present study was to compare the antioxidant capacities of the selected natural pigments relative to the synthetic antioxidant BHA (the control).

Several different methods are necessary to obtain reliable values of antioxidant capacity (Bernaert et al., 2012). Erythrocytes are the most abundant cells in the living body and possess desirable physiological and morphological characteristics. Oxidative stress mimicked by AAPH challenge in erythrocytes is expected to reflect the complexity of organisms more accurately than a simple chemical reaction. Therefore, in the present study, the antioxidant capacities of these natural pigments were first measured by using chemical assays (i.e., DPPH, $ABTS^{\cdot+}$, $O_2^{\cdot-}$, H_2O_2 and FRAP assays). Then, curcumin and cyanidin-3-rutinoside, which showed the best free radicals scavenging activities in the aforementioned assays, were further verified by the chicken erythrocyte model.

3.1. DPPH and $ABTS^{\cdot+}$ scavenging activity

DPPH, a stable nitrogen radical, has been widely used to evaluate radical quenching capacities of natural antioxidants and some other plant extracts. The addition of quenchers or reducers to the DPPH solution induces a rapid change from purple or violet color to yellow, which indicates the formation of a stable diamagnetic molecule with the optical density at 515 nm (Molyneux, 2004; Sharma & Bhat, 2009).

The capacities of seven natural pigments, assayed to be scavenging the DPPH radical in comparison with synthetic antioxidants BHA, were shown in Fig. 1. The scavenging ability of curcumin and cyanidin-3-rutinoside were significantly higher than other tested pigments and the BHA (control). At the concentration of 60 μg/ml, both the curcumin and cyanidin-3-rutinoside showed higher DPPH radical-scavenging activities, 98.69 ± 0.9% and 93.42 ± 0.9%, respectively. However, lycopene, β-carotene and betalain did not follow a dose-dependent manner. The DPPH values of lycopene, β-carotene and betalain were very low with few changes within the given concentration range (i.e., 10–60 μg/ml).

Müller et al. (2011) found that none of the analyzed carotenoids, including lycopene and β-carotene, showed ability to scavenge DPPH. Prolonging of the reaction time and changing the solvents did not lead to a decrease in the DPPH absorbance caused

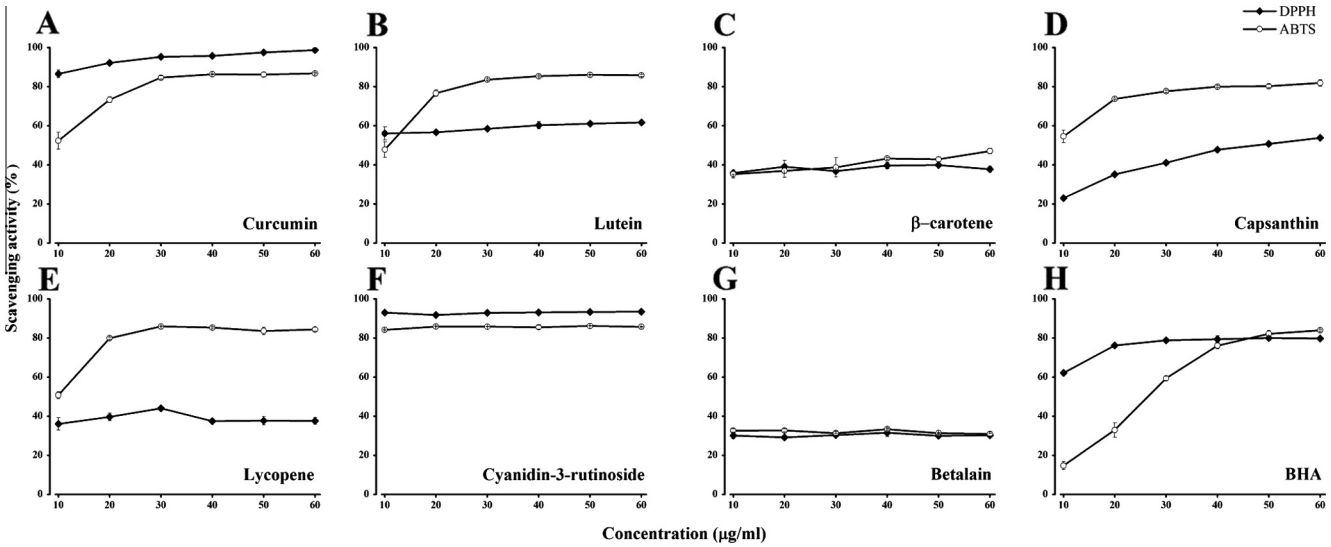

Fig. 1. DPPH and ABTS radical scavenging effects of (A) curcumin, (B) lutein, (C) β-carotene, (D) capsanthin, (E) lycopene, (F) cyanidin-3-rutinoside, (G) betalain, and (H) BHA at 10–60 μg/ml. Values of DPPH and ABTS scavenging ability are expressed as the mean ± SD of three independent experiments, respectively.

by the presence of carotenoids. Liu et al. (2008) determined the activity of β-carotene and lycopene using the similar reaction conditions, and found that the reaction of β-carotene and DPPH did not appear to be dose-dependent, since the lowest level of β-carotene did not produce the lowest scavenging capacity. Surprisingly, lycopene showed an inverse relationship between the scavenging capacity and concentration. It has been proposed that the measurable but low values of DPPH scavenging activity of β-carotene and lycopene may be due to the dark brown color generated by the mixture of carotenoid solution and DPPH solution, as this abnormal color may interfere with the absorbance reading at 515 nm (the optimum wavelength for DPPH measuring) (Molyneux, 2004; Sharma & Bhat, 2009). In the present study, the resulting absorbance value was measured at 517 nm, where DPPH still has significant absorbance.

ABTS is a peroxidase substrate, which generates a metastable radical with blue-green color through oxidation in the presence of H_2O_2 (Re et al., 1999). Therefore, the decolorization assay of $ABTS^{\cdot+}$ is shown to be a very useful tool in expeditiously measuring the antioxidative activity of individual chemical compounds or complex extracts. Among the lipid soluble pigments, curcumin showed the highest $ABTS^{\cdot+}$ scavenging effect while cyanidin-3-rutinoside appeared to be the best quencher compared with the water soluble pigments (Fig. 1). Curcumin had 52.38 ± 4.33% and 86.79 ± 0.34% radical scavenging ability whereas cyanidin-3-rutinoside had 84.21 ± 0.25% and 85.76 ± 0.15% scavenging ability at 10 and 60 μg/ml, respectively. The $ABTS^{\cdot+}$ scavenging activity of the seven natural pigments was ranked at the concentration of 40 μg/ml in decreasing order as follows: curcumin > cyanidin-3-rutinoside > lutein, lycopene > capsanthin > BHA > β-carotene > betalain. These results indicated that curcumin was a more potent $ABTS^{\cdot+}$ scavenger than the other natural pigments and BHA. With the exception of β-carotene and betalain, the remaining five natural pigments expressed much stronger inhibitory effects on $ABTS^{\cdot+}$ than BHA at the concentration ranging from 10 to 40 μg/ml.

DPPH and ABTS radicals are the two most widely used chromogenic compounds to measure the antioxidant activity of biological materials. Both of them were characterized by excellent reproducibility and stability under certain assay conditions (Arnao, 2000). However, they work through different mechanisms in their response to antioxidants. DPPH radicals are acquired directly after the work solution is prepared while $ABTS^{\cdot+}$ are generated by chemical reaction in the dark for 12 h. ABTS radical can be solubilized in both aqueous and organic media, in which the antioxidant activity can be measured due to the hydrophilic and lipophilic nature of the compounds in samples (Arnao, Cano, & Acosta, 1999). In contrast, DPPH can only be dissolved in organic media, which is a limitation when interpreting the role of hydrophilic antioxidants (Cano, Acosta, & Arnao, 2000). Moreover, the absorbance in $ABTS^{\cdot+}$ bleaching assay was recorded at 734 nm, at which the interference between carotenoids and the DPPH stock solution was negligible. Therefore, it was apparent that these natural pigments reacted more actively with $ABTS^{\cdot+}$ solutions than with DPPH solutions.

3.2. $O_2^{\cdot-}$ scavenging activity

$O_2^{\cdot-}$ is one of the most representative free radicals. In cellular oxidation reactions, $O_2^{\cdot-}$ is normally formed through a number of enzyme systems or non-enzymatic electron transfers, and its effects can be magnified because it produces other kinds of cell-damaging free radicals and oxidizing agents (Wang, Yuan, Jin, Tian, & Song, 2007). Hence, in the present study, a PMS–NBT assay was carried out to test the $O_2^{\cdot-}$ scavenging activities of seven natural pigments and BHA.

As shown in Table 1, each natural pigment possessed different activities and only lutein showed relatively lower activity than BHA. Cyanidin-3-rutinoside owned the highest scavenging activity of 80.47 ± 0.69% over the other natural pigments. The $O_2^{\cdot-}$ scavenging activity of seven natural pigments decreased in the order of cyanidin-3-rutinoside > curcumin > lycopene > betalain > β-carotene > capsanthin > BHA > lutein at a concentration 10 μg/ml. Similar with other *in vitro* studies, cyanidin-3-rutinoside has been proved to be the key antioxidant in many dietary plants (Kähkönen & Heinonen, 2003).

3.3. H_2O_2 scavenging activity

H_2O_2 is relatively an unstable metabolic product, which can penetrate biological membranes. Together with other ROS, H_2O_2 can give rise to hydroxyl radicals and singlet oxygen formed by Fenton reaction, and initiate lipid peroxidation. H_2O_2 itself is also harmful to several cellular components such as DNA, lipid and

Table 1
Effect of seven natural pigments (10 μg/ml) on $O_2^{\cdot-}$ production, H_2O_2 scavenging activity, and ferric reducing antioxidant power (FRAP).

Pigments	$O_2^{\cdot-}$ scavenging activity (%)	H_2O_2 scavenging activity (%)	FRAP (μmol $FeSO_4$/ml)
Curcumin	26.79 ± 0.97^{b}	53.83 ± 0.92^{b}	8.02 ± 0.59^{bcd}
Lutein	12.00 ± 1.54^{d}	45.64 ± 1.57^{c}	12.78 ± 0.06^{a}
β-Carotene	23.99 ± 3.07^{bc}	57.03 ± 0.50^{b}	3.96 ± 1.12^{be}
Capsanthin	21.73 ± 3.72^{bc}	52.09 ± 1.87^{b}	7.01 ± 0.21^{bce}
Lycopene	25.82 ± 3.35^{bc}	86.68 ± 1.27^{a}	9.14 ± 3.31^{acd}
Cyaniding 3-rutinoside	80.47 ± 0.69^{a}	82.25 ± 4.64^{a}	6.33 ± 0.35^{bce}
Betalain	23.81 ± 0.75^{bc}	86.98 ± 0.35^{a}	2.60 ± 1.67^{e}
BHA	19.36 ± 0.43^{c}	41.00 ± 0.27^{c}	3.64 ± 1.60^{be}

Each value is expressed as the mean ± SD ($n = 3$) of three independent experiments. Values in the same column with different lowercases were significantly different at $p < 0.05$.

nucleus (Heo, Park, Lee, & Jeon, 2005). Thus, the removal of H_2O_2 is very important for antioxidant defense in living systems.

Table 1 showed the percentage of H_2O_2 scavenging activity of seven natural pigments and BHA at 10 μg/ml. A significant increased scavenging activity of seven natural pigments was observed in the H_2O_2 scavenging assay as compared to the other assays, with several pigments yielding approximately 80% scavenging activity. Betalain and lycopene had the highest activity (approximately 86.98 ± 0.35% and 86.68 ± 1.27%, respectively) and cyanidin-3-rutinoside exhibited relatively lower scavenging activities (approximately 82.25 ± 4.64%). These results revealed that these scavenging effects of pigments were superior to those of BHA (around 41.00 ± 0.27%).

Generally, the methods to determine the total antioxidant capacity were divided into two major groups: assays based on the single electron transfer reaction, displaying oxidant reduction through a color change and assays based on a hydrogen atom transfer, measuring the activity of the antioxidant to scavenge peroxyl radicals (Huang, Ou, & Ronald, 2005). Herein, H_2O_2 scavenging assay based on electron transfer reaction belongs to the latter one. Thus, the ability of pigments to donate electrons and neutralize H_2O_2 to water might be well corroborated by the H_2O_2 scavenging activity. However, the exact mechanism by which the natural pigments inhibit H_2O_2 remains unknown.

3.4. FRAP assay

The FRAP assay appears to be an attractive and potentially useful assay among all the methods used for the measurement of oxidative stress and antioxidant defense system. The FRAP assay is quick and simple to perform. The reaction is reproducible and linearly related to a wide concentration range of the antioxidants present, and the equipment required is of a type commonly found in biochemical laboratories (Benzie & Strain, 1996).

Table 1 depicted the FRAP values of seven natural pigments compared with BHA. At 10 μg/ml, lutein and lycopene had significantly higher FRAP values of 12.78 and 9.14 μmol $FeSO_4$/ml, respectively. However, BHA exhibited only 3.64 μmol $FeSO_4$/ml of ferric reducing activity. The FRAP values of seven natural pigments decreased in the following order: lutein > lycopene > curcumin > capsanthin > cyanidin-3-rytinoside > β-carotene > BHA > betalain.

Of all the antioxidant capacity detection assays, the FRAP assay is the only assay that directly measures antioxidants in samples (Bernaert et al., 2012). The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric reaction, in which the redox potential of the antioxidants analyzed is important (Benzie & Strain, 1996). Therefore, the above results suggested that lutein had the strongest reducing capacity and lycopene would be the second best candidate among the tested pigments as well as BHA.

3.5. Protective effect of curcumin and cyanidin-3-rutinoside against AAPH-induced hemolysis

Erythrocytes, which possess high membrane concentration of polyunsaturated fatty acids and play a specific role as oxygen carriers, are considered to be the major targets for free radical attack (Ajila & Prasada Rao, 2008). Thus, besides cell free-system and chemical methods, we also employed the erythrocytes as a cell model to study the protective effects of natural pigments against oxidative insult. AAPH is an aqueous peroxyl radical initiator that generates free radicals by its thermal decomposition without any bio-transformations or enzymes. In the present study, AAPH was used as the free-radical initiator to induce oxidative injury in chicken erythrocytes. Moreover, the rate of radical generation is easily controlled by adjusting the concentration of initiators (Yuan, Wang, Yao, & Chen, 2005).

Based on the results of previous chemical assays used in the present study, we found that curcumin and cyanidin-3-rutinoside had the strongest free radical scavenging effects in DPPH and ABTS assays, and great ability in quenching $O_2^{\cdot-}$, H_2O_2 and reducing ferric ions. Thus, we selected these two pigments for further evaluation of the protective effects on hemolysis in AAPH-challenged chicken erythrocytes. Fig. 2 showed the effects of curcumin and cyanidin-3-rutinoside (0.5–10 μM) on erythrocytes exposed to the water-soluble radical initiator AAPH. Erythrocytes incubated in PBS buffer at 37 °C as a 2% suspension were stable with little hemolysis observed within 5 h (3.69 ± 0.15%). When AAPH was added to the suspension, hemolysis induction was increased in a time-dependent manner. Both the curcumin and cyanidin-3-rutinoside were shown to prevent the AAPH-challenged erythrocyte membrane from hemolysis in a concentration- and time-dependent manner. Moreover, curcumin showed a significantly ($p < 0.05$) higher inhibitory power of hemolysis (27.43–48.18%) than that of cyanidin-3-rutinoside (23.50–41.64%) from 0.5 to 10 μM for 5 h. When the erythrocytes were incubated with cyanidin-3-rutinoside or curcumin alone at the applied concentrations (0.5–10 μM), hemolysis was maintained at a background level similar to that of the AAPH untreated samples (data not shown).

Lipid-soluble antioxidants exert good antioxidant effects due to their hydrophobicity in the erythrocyte model. Banerjee, Kunwar, Mishra, and Priyadarsini (2008) found that when erythrocytes were preincubated with curcumin for 30 min, the majority of curcumin may have been taken up by the erythrocytes, leaving very minute amounts in the extracellular aqueous medium. Recent studies suggested that the ability of certain polyphenols to partition in cell membranes and the resulting restriction of their fluidity could sterically hinder the diffusion abilities of free radicals and thereby decrease the kinetics of free radical reactions (Singh & Rajini, 2008). In the present study, the erythrocytes were incubated with curcumin and cyanidin-3-rutinoside for 30 min prior to AAPH addition instead of being treated with pigments and AAPH

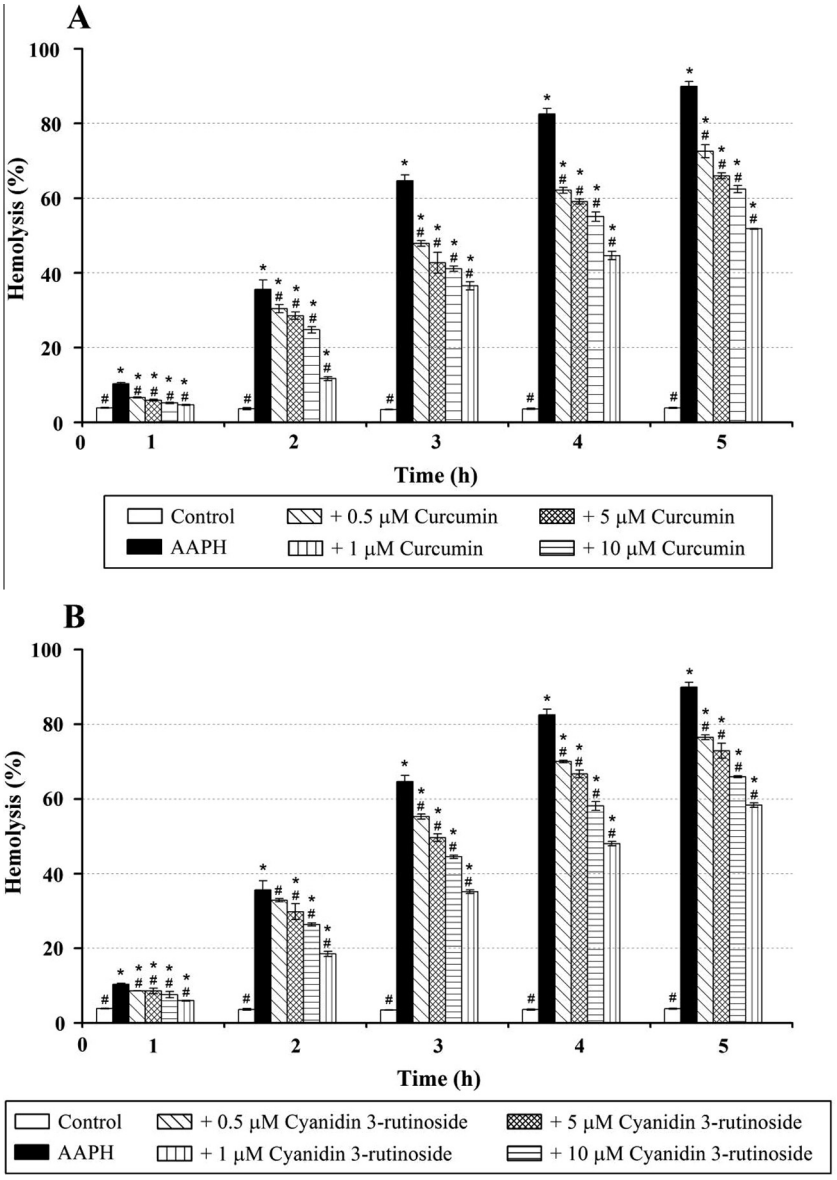

Fig. 2. Inhibition of AAPH-induced erythrocyte hemolysis by (A) curcumin and (B) cyanidin-3-rutinoside. The erythrocytes was preincubated with curcumin or cyanidin-3-rutinoside at the indicated concentrations for 30 min at 37 °C. The cell suspension was then incubated with 75 mM AAPH for 5 h at 37 °C. * Significant difference with control group ($p < 0.05$). #Significant difference with AAPH group ($p < 0.05$).

simultaneously. Curcumin, as a fat-soluble phenolic antioxidant, might function by incorporating itself into the hydrophobic regions of the membrane bilayer. Hence, the powerful protection of curcumin against hemolysis induced by AAPH in the present study might be explained by its hydrophobicity in biological chicken erythrocyte model.

3.6. Lipid peroxidation

Erythrocyte membrane lipids could lose a hydrogen atom from an unsaturated fatty acyl chain and initiate the lipid peroxidation that propagates as a chain reaction when they are subjected to oxidative stress (Mendes, de Freitas, Baptista, & Carvalho, 2011). Lipid peroxidation to erythrocyte membrane may disturb the structural lipid bilayer, alter the membrane permeability, disrupt ionic channels, and eventually lead to dysfunction of the whole erythrocytes (Lang, Lang, Lang, Huber, & Wieder, 2006). In this study, the extent of lipid peroxidation was assessed by measuring the formation of MDA content, a well-known carbonyl product of oxidative lipid damage.

When the erythrocytes were incubated with curcumin or cyanidin-3-rutinoside in the absence of AAPH, the MDA level was maintained at a background level similar to that of the AAPH-untreated samples (data not shown). The MDA level was significantly increased over time in the presence of 75 mM AAPH (351.45 ± 8.08 mmol/g Hb for 1 h and 387.89 ± 5.04 mmol/g Hb for 5 h, respectively), suggesting a state of considerable erythrocytic oxidative stress. As shown in Table 2, both curcumin and cyanidin-3-rutinoside significantly protected erythrocytes by inhibiting the formation of intracellular MDA in a dose-dependent manner. The treatment with curcumin and cyanidin-3-rutinoside at their highest concentration for 5 h reduced MDA levels by 54.77% and 38.22% in AAPH-challenged chicken erythrocytes, respectively. The results suggested that curcumin had better protective effects against lipid peroxidation and could significantly reduce the production of MDA in AAPH-challenged chicken

Table 2
Effect of curcumin and cyanidin-3-rutinoside on AAPH-induced increase in MDA levels and decrease in T-SOD activity of erythrocytes.

Experimental condition	MDA (mmol/g Hb) time (h)			T-SOD activity (U/g Hb) Time (h)		
	1	3	5	1	3	5
Control	351.45 ± 8.08[#]	376.59 ± 10.06[#]	387.89 ± 5.04[#]	1444.28 ± 28.12[#]	1159.30 ± 78.95[#]	1050.54 ± 6.96[#]
+AAPH	1105.15 ± 76.28[*]	1367.25 ± 40.58[*]	1522.83 ± 3.60[*]	661.76 ± 8.20[*]	544.73 ± 19.69[*]	402.68 ± 16.63[*]
+0.5 μM CRM + AAPH	1045.84 ± 13.51[*,#]	1203.35 ± 18.43[*,#]	1405.66 ± 20.96[*,#]	893.60 ± 8.37[*,#]	781.07 ± 12.58[*,#]	634.12 ± 4.12[*,#]
+1 μM CRM + AAPH	928.58 ± 5.24[*,#]	1042.10 ± 24.52[*,#]	1238.83 ± 22.77[*,#]	907.33 ± 13.13[*,#]	849.53 ± 7.55[*,#]	674.35 ± 14.57[*,#]
+5 μM CRM + AAPH	716.81 ± 3.03[*,#]	856.34 ± 9.66[*,#]	925.88 ± 8.97[*,#]	995.94 ± 6.44[*,#]	863.34 ± 2.43[*,#]	704.98 ± 13.98[*,#]
+10 μM CRM + AAPH	439.46 ± 7.60[*,#]	519.04 ± 55.66[*,#]	688.72 ± 12.76[*,#]	1110.42 ± 28.43[*,#]	891.15 ± 12.40[*,#]	753.03 ± 11.43[*,#]
+0.5 μM C3R+AAPH	1077.80 ± 16.25[*,#]	1284.05 ± 9.07[*,#]	1402.57 ± 4.83[*,#]	865.68 ± 5.42[*,#]	762.86 ± 5.77[*,#]	622.22 ± 13.51[*,#]
+1 μM C3R + AAPH	1003.05 ± 10.96[*,#]	1136.58 ± 11.52[*,#]	1335.93 ± 16.27[*,#]	884.48 ± 7.03[*,#]	803.89 ± 7.84[*,#]	652.79 ± 6.68[*,#]
+5 μM C3R + AAPH	831.03 ± 9.28[*,#]	959.21 ± 9.10[*,#]	1023.34 ± 6.29[*,#]	926.85 ± 3.59[*,#]	821.66 ± 2.15[*,#]	665.44 ± 9.19[*,#]
+10 μM C3R + AAPH	605.63 ± 13.99[*,#]	741.46 ± 5.44[*,#]	940.81 ± 9.61[*,#]	1017.50 ± 2.99[*,#]	868.00 ± 8.16[*,#]	698.79 ± 7.82[*,#]

The erythrocytes were preincubated at 37 °C for 30 min in the presence of curcumin or cyanidin-3-rutinoside at different concentrations (0.5–10 μM), and then the oxidant AAPH (75 mM) was added. Values of MDA level and SOD activity are expressed as the mean ± SD of three independent experiments.
[*] Significant difference with control group ($p < 0.05$).
[#] Significant difference with AAPH group ($p < 0.05$). Curcumin (CRM), cyanidin-3-rutinoside (C3R).

erythrocytes. This function is concomitant with the previous inhibitory effect of curcumin in the hemolysis assay.

Various studies have reported that many function groups, such as aromatic hydroxyl group and β-diketone group, are responsible for the ability of scavenging free radicals of natural antioxidants (Ak & Gulcin, 2008; Magalhaes et al., 2009). While cyanidin-3-rutinoside contains more than one phenolic function groups, Curcumin possess both phenolic and β-diketone functional groups on the same molecule. The differences of functional groups in the two compounds might be one of the reasons for more efficient chain propagating peroxyl radicals quencher effects that stops the lipid peroxidation in curcumin than in cyanidin-3-rutinoside in the aqueous phase.

3.7. Total antioxidant enzyme (T-SOD)

T-SOD is widely distributed in all body cells, and its activity is very high in erythrocytes. This enzyme protects cells by dismutating the highly reactive $O_2^{\cdot-}$ to a less reactive species, H_2O_2, and is very susceptible to oxidative inactivation. In this study, the activity of T-SOD in chicken erythrocytes was found to be decreased by 27.26% after 5 h of incubation in comparison with the baseline value (Table 2). When AAPH was added to erythrocytes, a time-dependent declining pattern in T-SOD activity was observed. When compared to the control group, there was a significant reduction ($p < 0.05$) of T-SOD activity from 661.76 ± 8.20 U/g Hb after 1 h to 402.68 ± 16.63 U/g Hb after 5 h incubation. Both curcumin and cyanidin-3-rutinosid significantly prevented reduction of T-SOD activity in AAPH-challenged erythrocyte, with curcumin being more effective (Table 2). These results are consistent with those reported by Deng, Chen, Zhou, Yang, and Liu (2006), Tedesco, Luigi Russo, Nazzaro, Russo, and Palumbo (2001), showing protective roles of curcumin and anthocyanins from oxidative stress in human erythrocytes. However, the results of T-SOD activities were inconsistent with our previous results of $O_2^{\cdot-}$ scavenging activities, which indicated cyanidin-3-rutinosid to be the more effective one. It might be explained by the variation between *in vitro* chemical system and intracellular environment. Preincubation of erythrocytes with cyanidin-3-rutinoside or curcumin alone maintained T-SOD activity close to the control value (data no shown).

3.8. Annexin V binding

Suicidal death of erythrocytes (eryptosis) is characterized by cell shrinkage, membrane scrambling, and phosphatidylserine (PS) exposure at the outer membrane leaflet. Eryptosis is triggered by erythrocyte injury after several stressors, including oxidative stress (Lang et al., 2006). In this study, we used AAPH, an aqueous peroxyl radical initiator, to induce erythrocyte apoptosis. Double-labeling method is often employed to detect apoptotic cells in suspension using flow cytometry. In this method, living cells are labeled green by annexin V-FITC and dead ones are stained red by propidium iodide (PI). Chicken erythrocytes underwent eryptosis when challenged by stressors as showed in human erythrocytes (Lang et al., 2006). PI, a nuclear dye, is membrane impermeable and is generally excluded from viable cells, so it can only be used for identifying dead cells through combining itself to DNA. However, chicken erythrocytes, which contains a nucleus, could become a better erythrocyte model to perform the double-labeling assessment instead of the human erythrocytes.

As shown on the representative dot plot (Fig. 3A and Table 3), more that 95% of untreated chicken erythrocytes showed Annexin V-negative/PI-negative staining at 2 h, indicating a normal PS asymmetry. At 5 h, the percentage of Annexin V-positive/PI-negative cells in control group was also very low (0.697 ± 0.02%) (Fig. 3B and Table 3). However, AAPH-treated erythrocytes were accompanied by a progressive time-dependent shifting of the cells to the lower right quadrant, indicating an appearance of apoptotic features. After the pretreatment with curcumin (10 mM) prior to AAPH challenge (75 mM), approximately 0.780 ± 0.020% of cells were Annexin V-positive/PI-negative and 0.115 ± 0.094% of cells were Annexin V-positive/positive after 2 h, suggesting AAPH-induced apoptosis was significantly inhibited by curcumin (Fig. 3). Exposure of erythrocytes to cyanidin-3-rutinoside (10 mM) before being challenged by AAPH also markedly decreased the number of Annexin V-binding erythrocytes, approximately 1.327 ± 0.097% of Annexin V-positive/PI-negative cells and 0.237 ± 0.092% of Annexin V-positive/positive cells for 2 h. When incubated for 5 h, there were 6.820 ± 0.200% of Annexin V-positive/PI-negative cells and 1.407 ± 0.274% of Annexin V-positive/PI-positive cells in curcumin-pretreated groups, while 9.300 ± 0.262% of Annexin V-positive/PI-negative cells and 1.763 ± 0.212% of Annexin V-positive/PI-positive cells in cyanidin-3-rutinoside pretreated groups, as compared to AAPH-treated group (23.200 ± 1.039% of Annexin V-positive/PI-negative cells and 1.220 ± 0.629% of Annexin V-positive/PI-positive cells).

Mammalian fetal erythrocytes and erythrocytes from amphibians, reptiles and birds contain a nucleus undergo typical apoptosis. However, the main components of the death machinery were not clearly defined in these different models (Sakamoto, Mima, Takahashi, & Tanimura, 1997; Sekizawa et al., 2000). In the present study, we employed chicken erythrocytes as a model to investigate

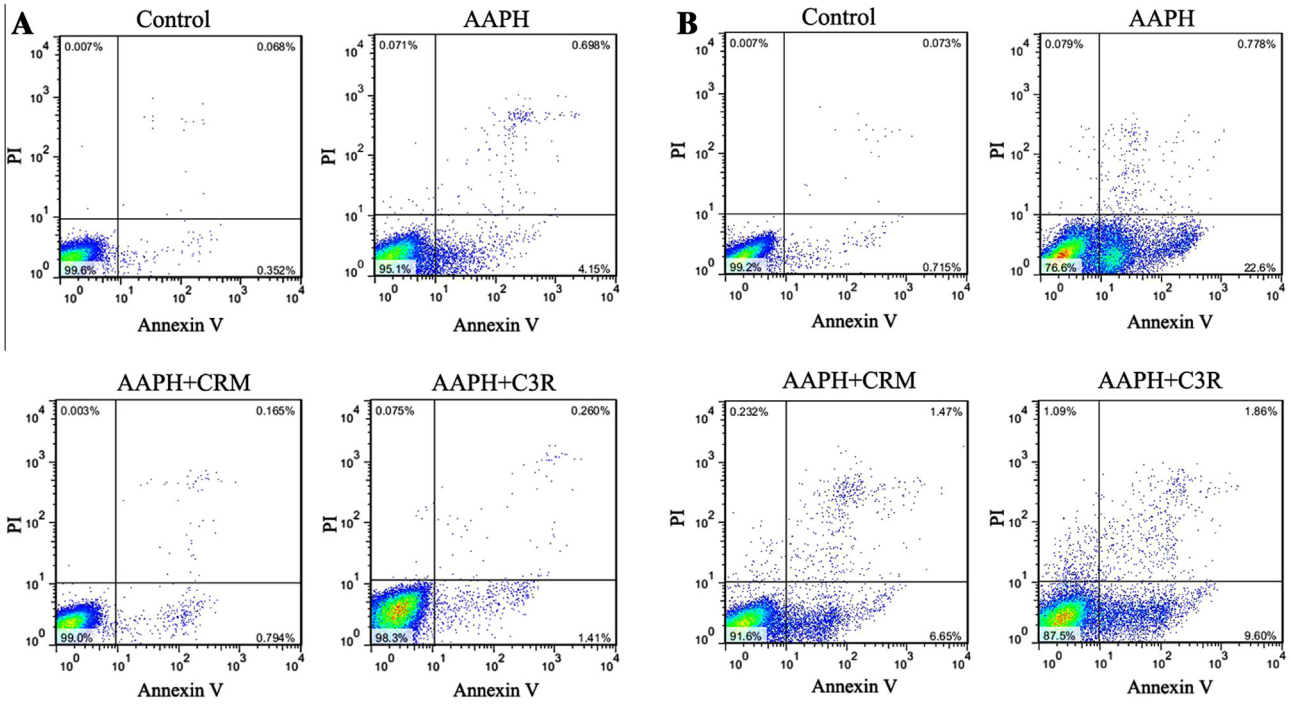

Fig. 3. Effect of curcumin and cyanidin-3-rutinoside (10 μM) on AAPH-induced erythrocytes apoptosis. Chicken erythrocytes were pretreated with curcumin or cyanidin-3-rutinoside at 10 μM, and then the oxidant AAPH (75 mM) were added. After incubation for (A) 2 h and (B) 5 h, the erythrocytes were collected and mixed with Annexin V-FITC and propidium iodide (PI) at 4 °C for 15 min, and analyzed using flow cytometry.

Table 3
Effect of curcumin and cyanidin-3-rutinoside (10 μM) on AAPH-induced chicken erythrocytes apoptosis.

Experimental conditions	2 h			5 h		
	Annexin V^+/PI^-	Annexin V^+/PI^+	Annexin V^-/PI^+	Annexin V^+/PI^-	Annexin V^+/PI^+	Annexin V^-/PI^+
Control	0.393 ± 0.092^{d}	0.067 ± 0.001^{b}	0.041 ± 0.055	0.697 ± 0.020^{d}	0.081 ± 0.007^{b}	0.010 ± 0.006^{a}
+AAPH	4.037 ± 0.121^{a}	0.449 ± 0.217^{a}	0.095 ± 0.082	23.200 ± 1.039^{a}	1.220 ± 0.629^{a}	0.081 ± 0.005^{a}
+10 μM CRM + AAPH	0.780 ± 0.020^{c}	0.115 ± 0.094^{b}	0.060 ± 0.099	6.820 ± 0.200^{c}	1.407 ± 0.274^{a}	0.135 ± 0.096^{a}
+10 μM C3R + AAPH	1.327 ± 0.097^{b}	0.237 ± 0.092^{ab}	0.123 ± 0.045	9.300 ± 0.262^{b}	1.763 ± 0.212^{a}	0.885 ± 0.338^{b}

Chicken erythrocytes were pretreated with curcumin or cyanidin-3-rutinoside (10 μM), and then the oxidant AAPH (75 mM) were added. After incubation for 2 h and 5 h, the erythrocytes were collected and mixed with Annexin V-FITC and propidium iodide (PI) at 4 °C for 15 min, and analyzed using flow cytometry. Each value is expressed as the mean ± SD ($n = 3$) of three independent experiments. Values in the same column with different lowercases were significantly different at $p < 0.05$. Curcumin (CRM), cyanidin-3-rutinoside (C3R).

the AAPH-induced eryptosis through double-labeling method. Our results showed that AAPH induced hemolysis and triggered eryptosis in the chicken erythrocytes. The initiation of "apoptosis" serves to remove injured erythrocytes prior to hemolysis and thus to reduce the release of hemoglobin into plasma. As shown in Fig. 1, curcumin and cyanidin-3-rutinoside significantly attenuated the hemolysis caused by AAPH challenge, which was consistent with our results of eryptosis. Curcumin and cyanidin-3-rutinoside exerted their anti-eryptotic activity mainly through the depletion of MDA and the elevation of T-SOD activity in chicken erythrocytes.

4. Conclusion

In conclusion, our results demonstrated that the seven selected natural pigments showed varying extent in antioxidant activities *in vitro*. Free radical scavenging assay suggested that the fat-soluble curcumin and the water-soluble cyanidin-3-rutinoside were proven to be strong free radical quenchers. Moreover, both curcumin and cyanidin-3-rutinoside at the applied concentrations reduced lipid peroxidation, improved the antioxidant enzyme activity, and attenuated apoptosis and hemolysis in the AAPH-challenged chicken erythrocytes. Overall, these findings supported natural phenolic pigments, especially curcumin and cyanidin-3-rutinoside, could be promising antioxidants in neutralization of various free radicals. Further experiments in different cell types and animal models are required to fully evaluate the antioxidative potential of natural pigments and to elucidate their exact mechanisms.

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