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Protective Effects of a Freeze-Dried Extract of Vegetables and Fruits on the Hydroxyl Radical-Mediated Oxidative Damage of DNA and Decrease of Erythrocytes Deformability

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Abstract

The protective effects of a freeze-dried extracts of vegetables and fruits (BauYuan; BY) on the hydroxyl radical-mediated DNA strand breakages and the structural integrity of human red blood cells (RBCs) were investigated. First, the supercoiled plasmid (pEGFP-C1) DNA was subjected to oxidation damage by an ascorbate-fortified Fenton reaction and the protective effects were analyzed by agarose gel electrophoresis. In the absence of BY extracts, exposure of the high-throughput ·OH-generating system (Fe^{2+} concentration > 1.0 μ*M*) caused a complete fragmentation of DNA. Supplementation of BY extract (1 mg/mL) to the plasmid DNA prior to the exposure could prevent it significantly. In contrast, as the plasmid exposed to a low-grade ·OH-generating system (Fe^{2+} < 0.1 μ*M*), the BY extract (1 mg/mL) provided an almost complete protection. Next, the cell deformabilities were measured to assess the protection effects of various BY extracts on human erythrocytes exposed to the oxidative insults. We found that both the aqueous extract and the organic solvent-derived extracts could strongly protect human RBCs from

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the reactive oxygen species (ROS)-mediated decrease in the deformability indices. The results implicated that the BY extracts could effectively protect the cell membrane integrity via scavenging ROS which enabling RBCs to maintain a balance of water content and surface area to prevent the drop of cell deformability.

Index Entries: Freeze-dried extract of vegetables and fruits; hydroxyl radical; oxidative damage; DNA; protective effects; deformability profile.

Introduction

Free radicals are known to play many important roles, such as the killing of bacteria during phagocytosis, the introduction of nitric oxide in the biosignaling process, and the breakdown processes of abnormal cells in apoptosis and necrosis *(1)*. However, the production of reactive oxygen species (ROS) such as superoxide, hydroxyl radical, hydrogen peroxide, and nitric oxide are the unavoidable byproducts from all aerobic respiration-dependent living systems, and their potential to cause oxidative damage is also enormous.

Fruits and vegetables are the major resources of vitamins, minerals, dietary fibers and a wide range of phytochemicals (second metabolites) which are critical to human health. Epidemiological studies have shown that a high consumption of fruits and vegetables is associated with decreased risk of various human cancers *(2)* and cardiovascular diseases *(3)*. Many efforts have also been made to determine the nutritional effects of extracts of specific foods such as garlic *(4)*, the anti-oxidation effects of extracts from green and black teas *(5)*, and the antitumor promoting activities of extracts of medicinal plants such as ginseng *(6)*. In this study, the anti-oxidant properties and the protective effects of the freeze-dried extract of vegetables and fruits were evaluated.

Materials and Methods

Procurement of BY Vegetable and Fruit Extracts

The freeze-dried extract of vegetables and fruits (Bau-Yuan [BY]) was supplied by Tsuan-Ron Biotech Corp. (Taiwan). The extract consists of over forty domestic vegetables and fruits, including fruits (papaya, banana, Japanese apricot, tomato, pineapple, watermelon, cantaloupe, round kumquat, mulberry, longan, guava, lemon, and carambola), vegetables (spinach, Chinese mustars, radish, sprouting broccoli, great burdock *[Arctium lappa]*, water convolvulus, bitter gourd, celery, rapeseed, and vegetable sponge *[Luffa acutangula]*), bean sprouts and rizoma (mungbean sprout, Adzuki bean sprout, soy bean sprout, early dwarf pea sprout, wheat sprout, clover sprout, sun flower sprout, potato, sweet potato, amorphophallus konjac, and East Indian lotus). Without using chemical fertilizers and pesticides

during the entire growth period, the vegetables and fruits were obtained from the contracted farmers to prepare the concentrated juice. After removal of debris, the extract was obtained through a sophisticated freeze-dry technology. The BY extract retained approx 3% by weight of the original materials. Several enzymes and vitamins contained in BY extracts were assayed. For example, protease, 7.1×10^3 U/g; amylase, 1.4×10^2 U/g; vitamin B1, 4.2 µg/g; vitamin B2, 2.7 µg/g; vitamin B3, 13.2 µg/g; and superoxide dismutase (SOD), 2.8×10^4 U/g. The extract (10% solution) had also been assayed for the possible residues of 79 popular agricultural chemicals and toxic compounds currently used in Taiwan. However, none of them was found within the detection limits by the Taiwan Agricultural Chemicals and Toxic Substances Research Institute.

The *Escherichia coli* strains used in this study was TOP 10F′ (genotype, F{*lacI*^{q}Tn10(Tet^R)}*mcr*A Δ(*mrr-hsa*RMS-*mcr*BC)ϕ80*lac*ZΔM15 Δ*lacX*74*deo*R*rec*A1*ara*D139Δ(*ara-leu*)7697*gal*U*gal*K*end*A1*nup*G.) (Invitrogen Corp.). The plasmid pEGFP-C1 is a pEGFP derivative with kanamycin resistance Clontech Corp.

Oxidative Damage to Plasmid DNA

Plasmid DNA was extracted with QIAamp kit (QIAgene Corp.) according to the manufacturer. The BY was dissolved with ddH_2O to obtain 10% (1 g/10 mL; 10^{-1}X) stock solution. The solution was centrifuged with microcon-100 (Millipore Co.) and followed by microcon-3 to remove molecules larger than 3 kDa. To induce the oxidative stress to the plasmid DNA, the Fenton reactions: $Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^- + Fe^{3+}$ were carried out *(5)*. The 20-µL assay solution contains 0.01% H_2O_2, 0.2 m*M* vitamin C and 300 ng DNA of plasmid pEGFP-C1. The concentration of Fe^{2+} was between 10 µ*M* and 10 n*M* and the concentrations of BY were adjusted in the range of 1 mg/mL to 0.001 mg/mL.

Protein Oxidation Assay

To evaluate the protective efficiency of the extract BY for protein oxidation, bovine serum albumin (BSA) was chosen as the target protein and dissolved in phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.4 in 1 L). Then, 100 µl BSA (10 mg/mL) solution was added to 900 µL Dubecco's PBS (PBS with 0.1 g $CaCl_2$ and 0.1 g $MgCl_2$ in 1 L, pH 7.2) containing 25 m*M* HEPES buffer, 25 m*M* vitamin C, $FeCl_3$ (0, 500 or 1000 µ*M*) and diluted BY(1 mg/mL to 0.01 mg/mL). After incubation at room temp for 16 h, 100 µL of 10 m*M* diethylenetriamine pentaacetic acid (DTPA) was added to stop the oxidative stress. To recover the BSA protein, the solution was dialyzed against 500 mL Dubecco's PBS/1 mM DTPA at 4°C for 2 h, followed by dialysis with Dubecco's PBS twice, and then the BSA concentration was adjusted with Dubecco's PBS to 3 mg/mL.

The BSA solution was precipitated with 100% TCA and then treated with 0.2% DNPH (2,4-dinitrophenyhydrazine)/2 *N* HCl, followed by 100% TCA. After centrifugation, the pallet was washed with ethanol:ethyl acetate (1:1 v/v). The protein was then dissolved in 6 *M* guanidine HCl (in 20 m*M* sodium phosphate buffer, pH 6.5) and OD_{370} was measured to estimate the content of carbonyl groups *(7)*.

Cell Survival Assay

The NIH3T3 cell line was cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The BY was dissolved with ddH_2O to obtain 10% (0.1 g/mL, 10^{-1}X) solution. This solution was centrifuged with microcon-100 (Millipore Corp.) and followed by microcon-3 to remove molecules larger than 3 kDa. The fraction was then 10-fold diluted to 0.01 g/mL with DMEM and filtered with a 0.22-µm filter. For the experimental group, cultured NIH3T3 cell were adjusted to 2×10^4 cells/mL suspension. H_2O_2 (4–50 m*M*) was added with or without BY (10^{-3} g/mL) on the next day. For the control group, the cell suspension was adjusted to 5000 cells/mL. After growing for two days, the cells were washed with PBS and mixed with 180 µl 10% DMEM and 20 µL MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt) solution. After growing for 4 h, OD_{490} was measured with the enzyme-linked immunosorbent assay (ELISA) reader (Dynex Technologies) to calculate the cell survival rate *(8)*.

Measurement of Deformability of Human Erythrocyte (RBC)

Blood samples (10 mL) were obtained from healthy volunteers and anticoagulated with heparin. The Ficoll-Paque ($D = 1.077$ g/cm^3) (Amersham Pharmacia Biotech) was used to separate the RBC from plasma and the puffy coat. RBCs were then washed with PBS to form packed RBC. The BY were dissolved in (1) ddH_2O, (2) 100% ethanol, (3) ethanol:Gethylacetate (1/1; w/w), or (4) 50% ethanol to form 10% (0.1 g/mL) solutions, and then centrifuged to obtain the supernatants. The reaction mixtures contained 100 µ*M* vitamin C, 0.1% H_2O_2, 100 µ*M* Fe^{2+}, 10% RBC, 4 m*M* sodium azide (dissolved in PBS) and one-tenth volume of BY extracted by different solvents. After shaking at 37°C for 2 h, the RBC were mixed with PVP buffer (PVP low [0.9 g Na_2HPO_4, 0.24 g NaH_2PO_4 and 31.0 g polyvinylpyrrolidone in 1 liter H_2O]:GPVP high [20 g NaCl in 500 mL PVP low buffer] = 3:1) and the deformability index (DI) was measured by the laser viscodiffractometer.

The osmotic deformability profiles of intact RBCs were determined by continuously monitoring the deformability index with a custom-built laser viscodiffractometer as a function of the osmolality of the medium (50 to 500 mOsm/kg) at a constant shear stress of 160 dynes/cm^2 *(9)*. In a typical run, 100 µL of whole blood was mixed with 4 mL of PVP buffer, which is a mixture of 37.5 mL of low-polyvinylpyrrolidone (PVP) buffer and 12.5 mL of high-PVP buffer.

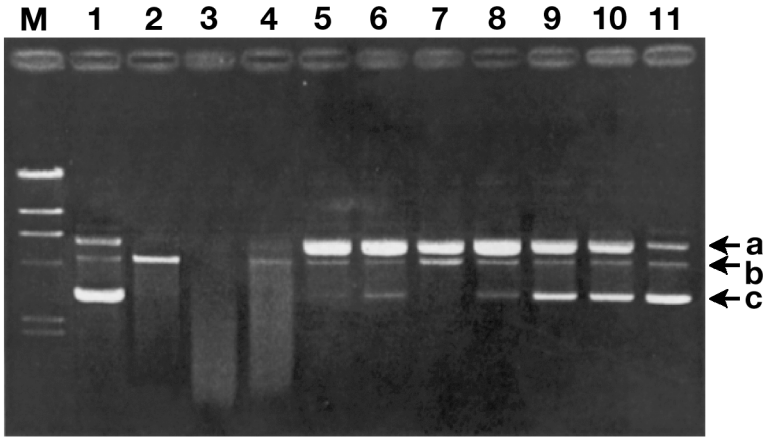

Fig. 1. Protection of oxidative damage of plasmid (pEGFP-C1) DNA by Bau Yuan (BY) extract (1 mg/mL) in the presence of an ascorbate fortified ·OH-generating system. Lane M, lambda *Hin*dIII DNA size marker, from the top: 23.0, 6.4, 4.4, 4.0, 2.2, and 2.0 kb. Lane 1, uncut plasmid pEGFP-C1 (4.7 kb) with varied supercoil density. Lane 2, pEGFP-C1 *Bam*HI digest. Lane 3-10, contains 0.01% H_2O_2, 0.2 m*M* ascorbate and varying concentrations of Fe^{2+} (10, 1, 0.1, 0.01, 10, 1, 0.1, and 0.01 µ*M*, respectively). Lanes 3–6, plasmid pEGFP-C1 treated with varying degrees of ·OH-generating system without added BY extracts. Lanes 7–10, same as in lanes 3–6, but with the presence of BY extracts (1 mg/mL). Lane 11, untreated and without the addition of BY extracts. The position of plasmid DNA: a, relaxed form (form II); b, linear form (form III); c, uncut plasmid (form I).

Results and Discussion

In recent years, there has been considerable interest in vegetables and fruits as health foods and in their natural resources against diseases and aging problems *(10–13)*. The BY extracts consist of not only more than 40 kinds of vegetables and fruits but also are free from chemical fertilizers and pesticides. The present study evaluated the potential antioxidant properties of BY at both the plasmid DNA and the cellular levels. Could the BY extract protect plasmid DNA from oxidative damage? In the present study, the aqueous fraction of BY extract was first chosen to check the protection capability for plasmid DNA under an oxidative stress. As indicated in Fig. 1, in the absence of BY extracts, exposure of pEGFP-C1 DNA to the ·OH-generating system (0.01% H_2O_2, 0.2 m*M* ascorbate and Fe^{2+} concentration > 1.0 µ*M*) caused a complete fragmentation of DNA (smear pattern). However, supplementation of BY extracts (1 mg/mL) prior to the exposure of the high-throughput ·OH-generating system, could prevent the pEGFP-C1 DNA from fragmentation and most of the DNAs were retained in form II (relaxed form) and form III (linear form). When pEGFP-C1 DNAs were exposed to low-grade OH-generating system (Fe^{2+} < 0.1 µ*M*) in the presence of BY extracts (1 mg/mL), a significant protection of DNA oxidative damage could also be seen as judged by the relative proportion of form I (supercoiled form), form II and form III (Fig. 1). Along this line, we have

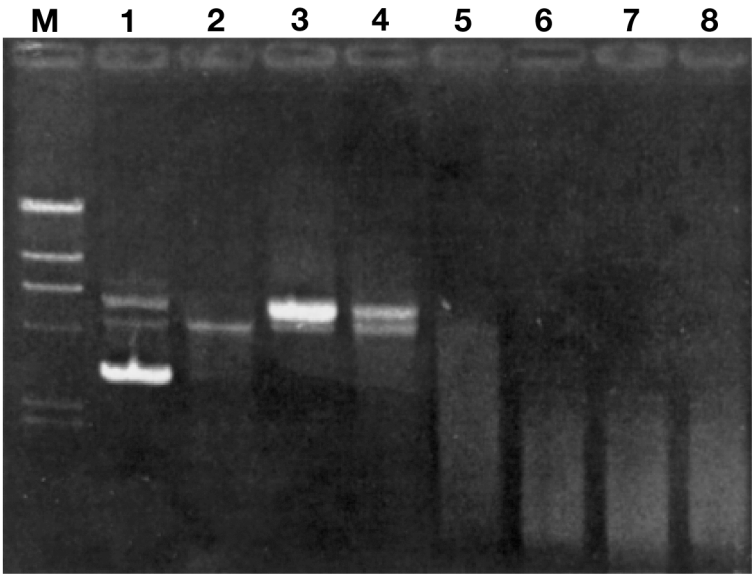

Fig. 2. Protection of oxidative damage of plasmid pEGFP-C1 DNA via an ascorbate fortified high-throughput ·OH-generating system (1 % H_2O_2 + 10 µM Fe^{2+} + 0.2 µ*M* ascorbate) by Bau Yuan (BY) extracts. Lane M, lambda *Hin*dIII DNA size marker, from the top: 23.0, 6.4, 4.4, 4.0, 2.2, and 2.0 kb. Lane 1, uncut plasmid pEGFP-C1 (4.7 kb). Lane 2, pEGFP-C1, *Bam*HI digest. Lane 3-8, plasmid pEGFP-C1 DNA with the added BY extracts of varied concentrations: 1.0, 0.5, 0.1, 0.05, and 0 mg/mL, respectively.

also demonstrated that the protective effect of BY extracts against this high-throughput ·OH-generating system was concentration-dependent (Fig. 2)

The BY extract was further examined for its potential to protect protein molecules from oxidative damage. Free carbonyl group, an index of protein peroxidation, and the extent of metal-catalyzed oxidation were measured as described in "Methods." Our results showed that under the stress (generated by 25 m*M* vitamin C, 25 m*M* HEPES buffer and 500 or 1000 µ*M* Fe^{3+}), the BY extract (1.0 or 0.01 mg/mL) could provide 20–30% protection to the proteins from oxidative damage (Fig. 3). The protective effects of the BY extract on the viability of cells under oxidative stress were also examined. However, no significant protection was observed (data not shown) possibly as a result of the complex factors involved in controlling cell viability.

Erythrocytes are very susceptible to oxidation by ROS. The overall cellular characteristics of human erythrocytes upon exposing to ·OH-generating system were therefore evaluated. We used a sensitive and reliable viscodiffractometric method to identify cell abnormalities in terms of cell water content or the membrane surface area *(4,14,15)*. As shown in Fig. 4, the RBC deformability profile reaches its peak at approx 295 mOsm/kg (isotonic region) under the normal circumstance and the treatment of hydrogen peroxide caused severe loss of deformability. Four preparations of the BY extracts: (a) aqueous portion of the extract, (b) ethanol extract,

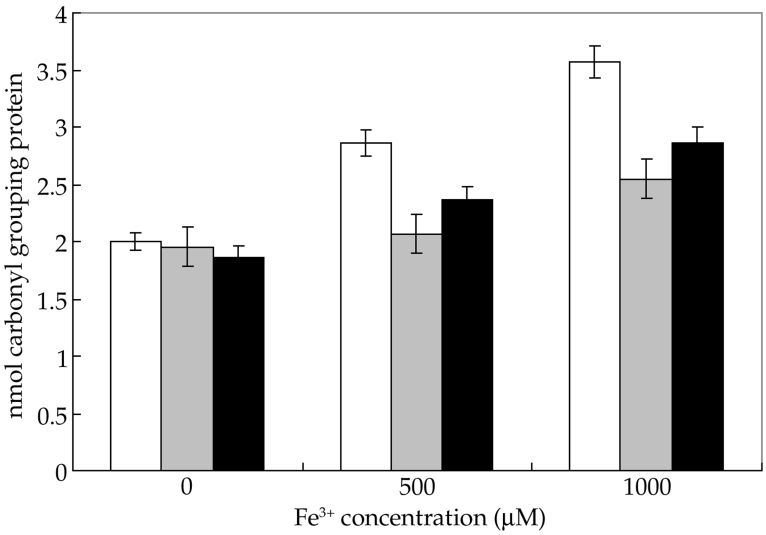

Fig. 3. Protection of protein carbonyl group formation by the vegetable and fruit extract Bau Yuan. Protein carbonyl group formation was triggered by the iron-ascorbate catalyzed oxidation as described in "Methods." The values of the blank, gray and dark columns represent the carbonyl contents induced by ferric ions, in the presence of 0, 0.01 mg/mL and 1 mg/mL of the BY extracts.

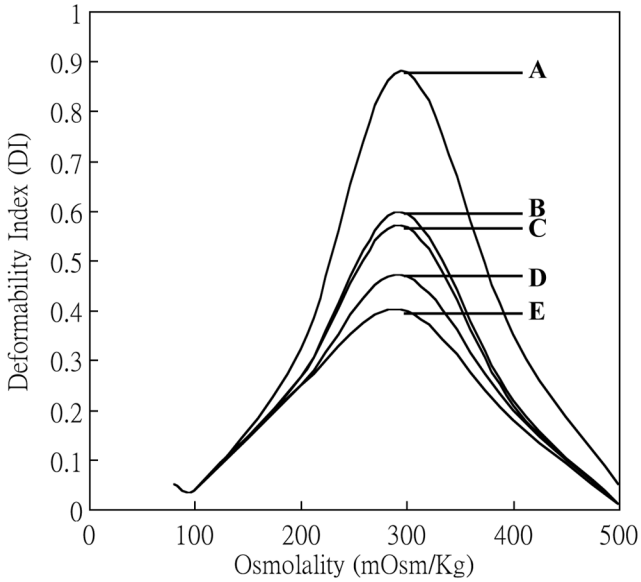

Fig. 4. The protective effects of aqueous Bau Yuan (BY) extracts on the osmotic deformability profile of human red blood cells. Curve **A**, the control experiment without exposing to an ascorbate-fortified ·OH-generating system (100 μ*M* Fe^{2+}, 100 μ*M* ascorbate and 1 m*M* H_2O_2). Curve **B**, treated with the ·OH-generating system, without the addition of BY. extract; Curve C, D and E, represents the RBC treated with the ·OH-generating system and in the presence of 0.5 mg/ml, 1.0 mg/ml, and 5.0 mg/ml BY extract.

Table 1
Protective Effects of Various Bau Yuan (BY) Extracts on the Decreases of Deformability Indices Induced by Ascorbate-Fortified ·OH-Generating System

	Deformability index (DI)				
Preparation of BY extracts	Untreaded control	T (·OH-treated)	T with BY (0.5 mg/mL)	T with BY (1.0 mg/mL)	T with BY (5.0 mg/mL)
Aqueous (A)	0.80	0.38	0.47	0.54	0.62
Ethanol (B)	0.75	0.40	0.45	0.50	0.52
Ethanol/ethyl acetate (1:1) (C)	0.78	0.45	0.48	0.50	0.54
50% ethanol (D)	0.72	0.40	0.42	0.56	0.54

(c) ethanol:ethylacetate (1/1, w/w) extract, and (d) 50% ethanol extract of BY, were evaluated for the protective effects on RBC deformability in the presence of an oxidative stress. The results, as shown in Table 1, demonstrated that all of the four BY extracts can protect human RBC against the loss of deformability in a concentration-dependent manner. Among the four extracts compared, the aqueous portion offered slightly better protection. It has been known that protease such as pepsin and papain common in many plant cells may interfere with the deformability of RBC. Therefore, human milk lysozyme, chicken egg white lysozyme, BSA and polylysine were chosen to examine their possible roles in RBC deformability. Our results demonstrated that none of the testing proteins has protection against the loss of deformability, except polylysine of 100 μ*M* (data not shown).

The present study evaluated the antioxidant properties of the vegetable and fruits extracts BY based on the criteria of oxidative damages to plasmid DNA, protein oxidation, cell viability and RBC deformability. The results indicated that the BY extracts provide significant protections against DNA oxidative damages and the decreases of the erythrocyte deformability in a concentration-dependent manner. This may offer the basis for a new strategy in treating various diseases and aging problems which may be etiologically due to the occurrence of oxidative stress. Vegetables, fruits, and other medicinal plants with antioxidant properties thus may have the potential to supplement the classical therapeutic interventions and used as adjuvant therapy *(16)*.

Acknowledgments

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