JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Relative Inhibition of Lipid Peroxidation, Cyclooxygenase Enzymes, and Human Tumor Cell Proliferation by Natural Food Colors

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The most abundant water soluble natural food colors are betacyanins and anthocyanins. Similarly, lycopene, bixin, β-carotene, and chlorophyll are water insoluble colors. Pure betanin, bixin, lycopene, chlorophyll, β-carotene, and cyanidin-3-*O*-glucoside were isolated from *Beta vulgaris*, *Bixa orellana*, *Lycopersicum esculentum*, *Spinacia oleracea*, *Daucus carrota*, and *Prunus cerasus*, respectively. These natural pigments, alone and in combination, were evaluated for their relative potencies against cyclooxygenase enzymes and tumor cell growth inhibition by using MCF-7 (breast), HCT-116 (colon), AGS (stomach), CNS (central nervous system), and NCI-H460 (lung) tumor cell lines. Among the colors tested, betanin, cyanidin-3-*O*-glucoside, lycopene, and β-carotene inhibited lipid peroxidation. However, all pigments tested gave COX-1 and COX-2 inhibition and showed a dose-dependent growth inhibition against breast, colon, stomach, central nervous system, and lung tumor cells, respectively. The mixtures of these pigments were also evaluated for their synergistic effects and chemical interactions at various concentrations. The mixture of anthocyanin and betanin negated their efficacy in the cell growth inhibitory assay and did not enhance the COX enzyme inhibitory activity. This is the first report of a comparative evaluation and the impact on biological activities of these pigments alone and in combination.

KEYWORDS: Betanin; cyanidin-3-O-glucoside; lycopene; bixin; β-carotene; chlorophyll; cell proliferation inhibition; antioxidant; COX-1; COX-2

INTRODUCTION

The color of food enhances its appeal to the consumer. The use of natural colors in food preparation is an ancient practice. Recently, consumers have been wary of synthetic colors due to their hazardous nature. For example, several countries have already prohibited the use of a number of synthetic food colorants. Most natural colors have health attributes in addition to their pleasing appearances. Therefore, natural colors are gaining significance not only for their brilliant appearances but also to enhance the health benefits of the products that it contains. There is a great demand for food and supplements containing an efficacious dose of these natural colors.

Most of the edible natural colors are either water soluble or lipid soluble. For example, betacyanins in beetroot, sugar beet, and *Amaranthus* spp., a popular leafy vegetable, and anthocyanins in numerous fruits such as cherry, strawberry, cranberry, grape, and blueberry are highly water soluble. Betacyanins and anthocyanins possess reddish cationic aglycones with distinct chemical structures. Other bright colors such as lycopene in tomato, bixin in annatto seeds, β-carotene in carrot, and chlorophyll in green produce are only soluble in nonaqueous medium.

The beetroot (*Beta vulgaris* L.) contains the water soluble red pigment betanin, 5-*O*-β-glucoside of betanidin. It has been used as a natural food color for centuries and as a folk medicine (*1*). Betacyanins and anthocyanins are not found together in the same tissue of the plant although they are found in flowers and fruits of the same plant (*2*). Anthocyanin colors range from yellow-orange to red-blue, depending on the pH of the plant tissue. Several studies have shown that anthocyanins exhibit antioxidant and antiinflammatory activities (*3*, *4*).

Among lipid soluble pigments, lycopene is one of the major carotenoids found in tomatoes (*Lycopersicum esuculentum*). It is known for its ability to prevent cancer and other cardiovascular diseases and, therefore, is a popular food colorant as well as a dietary supplement (*5*). Another important lipophillic natural color, used as a coloring agent in butter, margarine, corn oil, dairy products, macaroni, sausage, and cheese, is the carotenoid bixin from annatto (*Bixa orellana*) seeds (*6*). Epidemiological studies have indicated that the consumption of carotenoid-rich fruits and vegetables is associated with a lower incidence of

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10.1021/jf051399j CCC: $30.25
Published on Web 10/22/2005

cancer and cardiovascular diseases. Another carotenoid of great significance is β-carotene, and it exhibits potent antioxidant, cyclooxygenase, and cell proliferation inhibitory activities on selected human cancer cell lines (*7*).

The green pigment chlorophyll is abundant in leafy vegetables. For example, the spinach (*Spinacia oleracea*) contains as much as 1% of chlorophylls on a dry weight basis. Experiments have shown that the consumption of chlorophyll could prevent or delay the onset of certain types of cancers (*8*). In the present study, we have evaluated the relative efficacies of betanin, anthocyanin, lycopene, bixin, β-carotene, and chlorophyll alone and in combination as inhibitors of lipid peroxidation, cyclooxygenase enzymes, and human tumor cell proliferation. This is the first report of their relative potencies and the interactions when assayed as a mixture.

MATERIALS AND METHODS

The ^{1}H and ^{13}C NMR spectra were recorded on Varian 500 and 125 MHz spectrometers using $CDCl_3$, CD_3OD, and D_2O/DCl solutions. Chemical shifts are in δ (parts per million) relative to $CDCl_3$ at 7.24 ppm, CD_3OD at 3.31 ppm, and D_2O at 4.81 ppm for ^{1}H NMR and $CDCl_3$ at 77.23 ppm and CD_3OD at 49.15 ppm for ^{13}C NMR. The solvents used were of ACS reagent grade. Roswell Park Memorial Institute-1640 (RPMI-1640) medium and fetal bovine serum (FBS) were purchased from GibcoBRL (Grand Island, NY). MTT [3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide], doxorubicin (Adriamycin), dimethyl sulfoxide (DMSO), and phosphate buffer saline (PBS) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). SF-268 [tumor origin, central nervous system (CNS)], MCF-7 (tumor origin, breast), and NCI-H460 (tumor origin, lung) were purchased from the National Cancer Institute (NCI, Bethesda, MD). AGS (tumor origin, stomach) and HCT-116 (tumor origin, colon) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The cyclooxygenase (COX)-1 enzyme was prepared in our laboratory from ram seminal vesicles purchased from Oxford Biomedical Research, Inc. (Oxford, MI), and the COX-2 enzyme was from prostaglandin endoperoxide-H synthase-2 (PGHS-2)-cloned insect lysate. Butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), ibuprofen, aspirin, and naproxen were purchased from Sigma-Aldrich Chemical Co. Vioxx was provided by Dr. Subash Gupta at Sparrow Hospital (Michigan).

Plant Materials. Fresh beetroots, spinach, and tomatoes were purchased from Meijer Supermarket (Okemos, MI) and *B. orellana* seeds were purchased from Kingston, Jamaica, West Indies.

Extraction and Isolation of Pigments. Beetroots (1.16 kg) were chopped, blended, and boiled with water. After centrifugation, the supernatant was lyophilized to afford a dark red powder (138.9 g). An aliquot of this (9 g) was adsorbed on XAD-16 resin and eluted with acidified methanol (pH 3). The removal of solvent under vacuum afforded betacyanins as a dark powder (1.9 g). An aliquot of this (1.2 g) was dissolved in H_2O (4 mL) and fractionated by using C-18 medium-pressure liquid chromatography (MPLC) (30 mm × 350 mm). The column was eluted with a MeOH−acidic H_2O (pH 5) gradient starting with 10% MeOH. The fractions were collected by a fraction collector at 2 min intervals. The flow rate was 3 mL/min. At the end of 15 fractions (6 mL each), the MeOH concentration was increased by 10% until 100% MeOH. Six fractions collected at 20% methanol and deep red in color were evaporated to dryness and yielded pure betanin (35 mg) and were stored at −20 °C until analysis. The spectral data of this betanin were recorded in D_2O/DCl. The ^{1}H and ^{13}C NMR data of betanin isolated were identical to the published data of betanidin-5-*O*-β-glucoside (*9*).

The powdered annatto seeds (600 g) were sequentially extracted in a glass column with hexane (3 × 2 L), chloroform (3 × 2 L), and methanol (3 × 2 L), and removal of the solvent under reduced pressure afforded 3, 50, and 11 g of extracts, respectively. An aliquot of the chloroform extract (1.2 g) was fractionated on a Silica gel column MPLC using hexane−acetone (90:10, 80:20, 70:30, and 50:50 v/v, 300 mL each) as solvent systems. The fractions collected at 70:30 hexane−acetone were concentrated at reduced pressure and yielded bixin (29 mg). It was further purified by preparative silica gel thin-layer chromatography (TLC) (500 μm) using hexane−acetone (6:4 v/v) as the mobile phase and gave a single reddish band. Elution of this band with CHCl3-MeOH (1:1) afforded pure bixin (23 mg). The ^{1}H and ^{13}C NMR data of the isolated bixin in $CDCl_3$ were found to be identical to the published spectral data of *trans*-bixin (*10*).

The spinach leaves (450 g) were blended with water, and the puree was centrifuged (model RC5C, Sorvall Instruments, Hoffman Estates, IL) at 10000*g* for 20 min at 4 °C. The resulting pellet was exhaustively extracted with methanol (3 × 2 L) in a glass column, and removal of the solvent under reduced pressure afforded a green extract (20 g). An aliquot of this extract (10 g) was stirred with hexane to give hexane soluble and insoluble portions. The hexane soluble portion was concentrated to dryness (6 g), and an aliquot (1.7 g) was fractionated by silica gel column (30 × 350) MPLC using hexane, hexane−acetone (95:5; 90:10, and 80:20 v/v) solvent systems as mobile phases. The fractions were collected at 2 min intervals using a fraction collector. The fractions collected at a 90:10 ratio of hexane−acetone were combined based on their TLC profiles and evaporated to dryness under reduced pressure to yield pure chlorophyll (25 mg). It was stored at −20 °C until the bioassays.

Fully ripened tomatoes (1.1 kg) were chopped, and the seeds and pulp were removed. The skin was blended with water and centrifuged (model RC5C, Sorvall Instruments) at 10000*g* for 20 min at 4 °C. The pellet was sequentially extracted in a glass column with methanol (3 × 3 L), ethyl acetate (3 × 3 L), and hexane (3 × 3 L), and removal of solvent under reduced pressure afforded 17, 30, and 11 g of extracts, respectively. An aliquot of the ethyl acetate extract (1.3 g) was fractionated by using silica gel column (30 × 350) MPLC using hexane−acetone (95:5, 90:10, 80:20, 70:30, and 50:50 v/v) solvent systems as the mobile phase. Fractions were collected at 3 min intervals by using a fraction collector. Seven fractions collected using 80:20 hexane−acetone mobile phase were evaporated under reduced pressure to yield impure lycopene (40 mg). It was further purified by preparative silica TLC (500 μm) using hexanes−ethyl acetate (70:30 v/v) as the mobile phase to afford pure lycopene (34 mg). It was stored at −20 °C until bioassays. The ^{1}H and ^{13}C NMR spectral data of the isolated lycopene were in agreement with the literature values of lycopene (*11*, *12*). The cyanidin-3-*O*-glucoside and β-carotene used in this study were pure samples isolated from natural sources earlier and stored in our laboratory at −20 °C.

Cancer Cell Growth Inhibitory Assay. The cancer cell proliferation inhibitory activity of all natural pigments isolated (**Figure 1**) was evaluated by using the MTT colorimetric assay. The tumor cell lines AGS, HCT-116, MCF-7, NCI-H460, and SF-268 used were maintained in our laboratory at Michigan State University. The cell lines were maintained as adherent cell cultures in RPMI-1640 medium supplemented with 10% FBS, 10 units of penicillin, and 105 μg/mL streptomycin at 37 °C under 5% CO_2 in a humidified incubator. The cells were harvested and counted by using a hemacytometer, and the appropriate number of cells (4000−8000 cells/mL) was transferred into 96 well plates and incubated for 24 h prior to the treatment. Betanin and cyanidin-3-*O*-glucoside were dissolved in water separately and diluted with RPMI-1640 medium to the desired concentrations of 12.5, 25, 50, 100, and 200 μg/mL. Lycopene, bixin, β-carotene, and chlorophyll solutions were prepared separately by dissolving them in DMSO followed by dilution with RPMI-1640 medium to yield final concentrations of 3.75, 7.5, 15, 30, and 60 μg/mL, respectively. The final concentration of DMSO was 0.2% in solvent control and treatments. Solutions of lycopene, bixin, β-carotene, and chlorophyll were mixed at their LD_{50} concentrations of 10, 30, 30, and 10 μg/mL, respectively. The synergistic effect of the mixtures was evaluated against all of the cell lines studied. The test compounds were exposed to cells for 48 h. Adriamycin (doxorubicin), dissolved in 0.1% DMSO, was used as the positive control. It was tested at 0.0905, 0.181, 0.363, 0.725, and 1.45 μg/mL. Water and DMSO (final concentration at 0.2% in supplemented RPMI-1640 media) were solvent controls. After incubation of test samples with cancer cells (48 h), a 25 μL aliquot of MTT solution (5 mg MTT per milliliter PBS solution) was added into each well and incubated for 3 h at 37 °C. The media, MTT, and dead cells

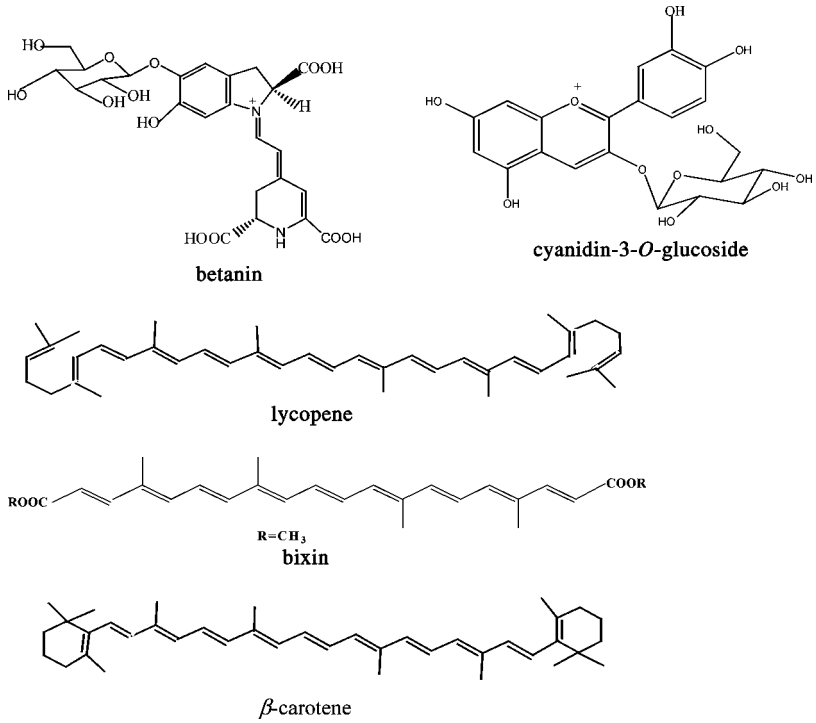

Figure 1. Chemical structures of betanin, cyanidin-3-*O*-glucoside, lycopene, bixin, and β-carotene.

were aspirated, 200 μL of DMSO was added to each well, and the plates were shaken for 5 min to dissolve the purple formazan crystals that formed. The absorbance was then measured at 570 nm with an automated microplate reader (model Elx800, Bio-tek Instruments, Inc., Winooski, VT). The experiment was conducted in triplicate for each concentration, and three parallel experiments were performed. The amount of formazan blue formed was proportional to the number of viable cells. The cell viability of samples at each concentration was calculated with respect to solvent control. The cell viability at each concentration was calculated by dividing the optical density of samples with the optical density of solvent control.

COX Enzyme Inhibitory Assay. COX enzyme inhibitory activities of natural colors were evaluated by using COX-1 and COX-2 enzymes. The rate of oxygen consumption during the initial phase of the enzyme-mediated reaction with arachidonic acid as the substrate was measured using a model 5300 biological oxygen monitor. The reaction mixture, consisting of 0.1 M Tris, 1.0 mM phenol, 17 μg of hemoglobin, the enzyme, and 10 μL of compound dissolved in DMSO at 1.5% DMSO (DMSO alone as solvent control), was held in a 600 μL microoxygen chamber at 37 °C. After 3 min of incubation, 10 μL of arachidonic acid was added to initiate the enzyme reaction. Data were recorded using quickLog for Windows. Aspirin (180 μg/mL), naproxen (10 μg/mL), ibuprofen (10 μg/mL), and Vioxx (1.67 μg/mL) were used as positive controls.

Lipid Peroxidation Assay. This assay was conducted by the analysis of a model liposome oxidation using fluorescence spectroscopy according to the procedure reported previously from our laboratory (*3*, *4*). The peroxidation was initiated by the addition of $FeCl_2$ for positive controls (BHA, BHT, and TBHQ, all 10 μM) and test samples. The fluorescence was measured at 384 nm and monitored at 0, 1, 3, and every 3 min thereafter up to 21 min using a Turner model 450 digital fluoremeter (Barnstead Thermolyne, Dubuque, IA). The decrease of relative fluorescence intensity with time indicated the rate of peroxidation, and the data were reported for 21 min after the initiation of peroxidation. The relative fluorescence was calculated by dividing the fluorescence value by their concentrations. The inhibition of lipid peroxidation by anthocyanins was reported from our laboratory (*3*, *4*); hence, cyanidin-3-*O*-glucoside was not assayed in this study.

Synergistic Studies of Pigments. The pigments evaluated for their synergistic cell proliferation inhibitory activities were betanin, anthocyanin, bixin, lycopene, β-carotene, and chlorophyll. The concentrations of betanin, anthocyanin, bixin, lycopene, β-carotene, and chlorophyll selected for the synergistic studies were 150, 150, 30, 10, 30, and 10 μg/mL, respectively. The combinations of pigments prepared for the study were (1) bixin + β-carotene + lycopene, (2) bixin + β-carotene + lycopene + chlorophyll, (3) bixin + β-carotene + lycopene + betanin + cyanidin-3-*O*-glucoside, (4) bixin + β-carotene + lycopene + chlorophyll + betanin + cyanidin-3-*O*-glucoside, and (5) betanin + cyanidin-3-*O*-glucoside. These combinations, 1−5, represented carotenoids, carotenoids + chlorophyll, carotenoids + water soluble betanin and anthocyanin, carotenoids + chlorophyll + water soluble betanin, and water soluble betanin + anthocyanin, respectively.

RESULTS AND DISCUSSION

The lipid peroxidation inhibitory activity of betanin was evaluated at 100 μg/mL solution in water. The anthocyanin, cyanidin-3-*O*-glucoside, was not tested in this assay since we have already reported its lipid peroxidation inhibitory activity (*3*, *4*). However, cyanidin-3-*O*-glucoside and betanin were assayed at 100 μg/mL as solutions in water. The lipid soluble lycopene and β-carotene were tested at 50 μg/mL, whereas bixin and chlorophyll were tested at 25 μg/mL as solutions in DMSO. The antioxidants BHT, BHA, and TBHQ were used as positive controls at 1 ppm. Betanin, lycopene, and β-carotene inhibited lipid peroxidation by 71, 82, and 73%, respectively (**Figure 2**). Both bixin and chlorophyll interfered with the assay; hence, lipid peroxidation inhibitory data are not available for these compounds. The water soluble betanin showed 71% of inhibition at 100 ppm although lipid soluble lycopene and β-carotene were equally active at 50 μg/mL. The higher antioxidant activity of lipophilic pigments is noteworthy since their bioavailability in human plasma may be enhanced by binding to lipoproteins.

Cyclooxygenase enzymes, COX-1 and COX-2, catalyze the conversion of arachidonic acid to generate chemical mediators of inflammation. Antiinflammatory drugs, ibuprofen (2.52 μg/mL), aspirin (180 μg/mL), Vioxx (1.67 μg/mL), and Celebrex (1.67 μg/mL), used as positive controls, gave 51, 40; 78, 99; 63, 32; and 0.7, 82% of COX-1 and COX-2 enzymes inhibition, respectively. Betanin and cyanidin-3-*O*-glucoside at 100 μg/mL and lycopene, chlorophyll, β-carotene, and bixin at 50 μg/

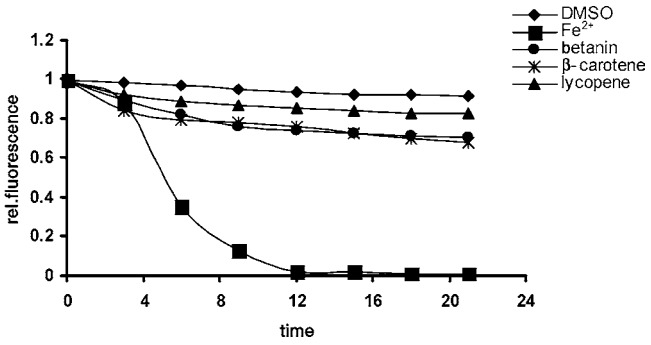

Figure 2. Lipid peroxidation inhibitory activity of betanin, lycopene, and β-carotene assayed at 100, 50, and 50 μg/mL, respectively. Commercial antioxidants BHA, BHT, and TBHQ were assayed at 1.66, 2.2, and 1.8 μg/mL, respectively. The peroxidation was monitored by the measurement of decrease in the intensity of fluorescence from 0 to 21 min. DMSO is the solvent control, and Fe+2 is the control oxidant.

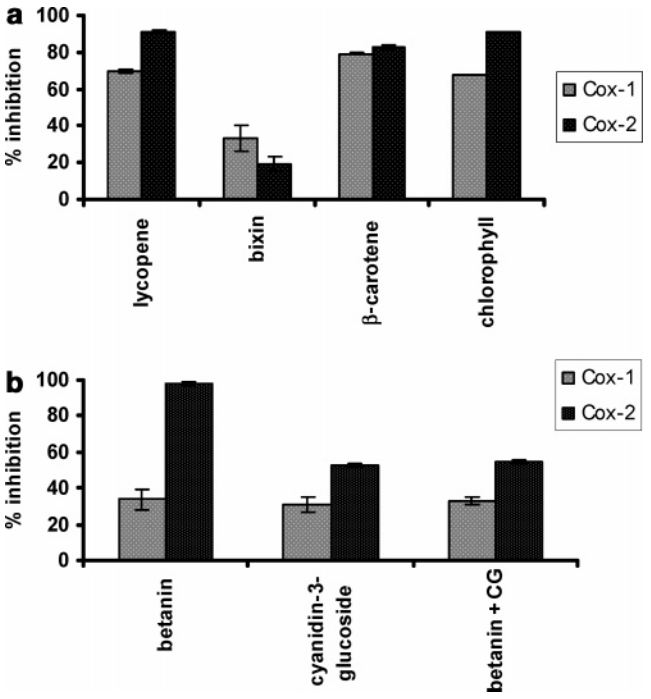

Figure 3. COX-1 and COX-2 enzyme inhibitory activities of (**a**) lycopene (50 μg/mL), bixin (50 μg/mL), chlorophyll (50 μg/mL), and β-carotene (50 μg/mL) and (**b**) betanin (100 μg/mL), cyanidin-3-*O*-glucoside (100 μg/mL), and a mixture of betanin + cyanidin-3-*O*-glucoside (100 μg/mL). The vertical bars represent the standard deviation of each data point ($n = 2$).

mL inhibited COX-2 enzyme by 97, 59, 90, 90, 82, and 19%, respectively. Similarly, betanin, cyanidin-3-*O*-glucoside, and bixin inhibited COX-1 enzyme by 33.5, 29.5, and 33.6% and lycopene, β-carotene, and chlorophyll by 69, 78, and 67%, respectively (**Figure 3a**,**b**). The mixture of betanin and cyanidin-3-*O*-glucoside, at 100 ppm each, was evaluated for COX enzyme inhibitory activity. Although betanin was selective against COX-2 enzyme, the mixture with anthocyanin reduced its original selectivity and inhibitory activity (**Figure 3b**).

These natural pigments were tested in a dose-dependent manner on breast, colon, stomach, CNS, and lung tumor cell lines for their ability to inhibit the cell growth. To determine their 50% growth inhibitory (IC_{50}) values, the hydrophilic pigments were tested at 12.5, 25, 50, 100, and 200 μg/mL and the lipophilic pigments at 3.75, 7.5, 15, 30, and 60 μg/mL, respectively (*13*). Betanin showed excellent growth inhibition of breast, colon, stomach, CNS, and lung cancer cell lines with IC_{50} values of 162, 142, 158, 164, and 147 μg/mL, respectively (**Figure 4a**). In our earlier reports, anthocyanins were assayed in DMSO solutions. In this study, the anthocyanin cyanidin-3-*O*-glucoside, the most abundant anthocyanin found in fruits, was assayed in water similar to betanin. The results showed a potent inhibition of the growth of lung cancer cells by cyanidin-3-*O*-glucoside with an IC_{50} value of 158 μg/mL. The IC_{50} values of growth inhibition for colon and breast cancer cell lines were 40 and 25%, respectively (**Figure 4b**). However, the growth of CNS cancer cells was not affected by cyanidin-3-*O*-glucoside at the concentrations tested.

The cell proliferation inhibitory effects of bixin, lycopene, β-carotene, and chlorophyll varied among tumor cell lines (**Figure 4c**−**f**). Among lipid soluble pigments, chlorophyll showed the highest growth inhibitory effects between 60 and 80% on all cell lines tested (**Figure 4f**). Bixin gave IC_{50} values at 33, 49, 45, and 39 μg/mL against colon, CNS, stomach, and lung cancer cell lines, respectively (**Figure 4c**), whereas lycopene gave 50% growth inhibition of colon, breast, and stomach cancer cell lines at 9, 22, and 30 μg/mL, respectively (**Figure 4d**). Lycopene had less impact on the growth of CNS and lung cancer cell lines, and the growth inhibitions were 35 and 25% at 60 μg/mL. At 45 μg/mL, β-carotene gave IC_{50} values of 26, 43, and 43%, respectively, against stomach, CNS, and colon cancer cell lines (**Figure 4e**). It inhibited the proliferation of lung and breast cancer cell lines by 30 and 45% at 60 μg/mL. However, chlorophyll showed 50% growth inhibition of breast, colon, stomach, CNS, and lung cancer cell lines at 18, 11, 12, 16, and 13 μg/mL, respectively (**Figure 4f**).

The synergistic effect of betanin, anthocyanin, bixin, lycopene, β-carotene, and chlorophyll is of great interest since foods containing these pigments are consumed in combination. Because bixin and chlorophyll interfered with the lipid peroxidation assay, they were not assayed for synergistic antioxidant activity. Similarly, the synergistic inhibitory activities of COX enzymes were not evaluated for the carotenoids and chlorophyll due to their weak or lack of COX-2 selectivity (**Figure 3**). On the basis of the dose−response results of the pigments evaluated in the cell proliferation assay (**Figure 4a**−**f**), these pigments were evaluated for their synergistic cell proliferation inhibitory activities.

Results indicated that not all combinations were as effective as the component pigments. The colon cancer cell lines were inhibited by 44, 55, 47, and 17%, respectively, when treated with 1, 2, 4, and 5 (**Figure 5a**). However, combinations 1−5 inhibited the growth of breast cancer cells by 37, 44, 42, 53, and 53%, respectively (**Figure 5b**). Although marginal, only pigment combinations 1, 2, and 4 showed growth inhibitory activity against lung cancer cells by 12, 30, and 17%, respectively (**Figure 5c**). The cell proliferation inhibitory activity was marginal for all combinations against CNS tumor cells (**Figure 5d**). The pigment combinations 1−4 were the best in inhibiting the growth of stomach cancer cell lines, as indicated by 55, 60, 37, and 55% inhibitions, respectively (**Figure 5e**).

In the lipid peroxidation inhibitory assay, bixin and chlorophyll interfered with the assay due to their strong absorption at the assay wavelength. Also, these compounds precipitated in the assay medium. Our earlier reports on the biological activities of anthocyanins were for DMSO solutions (*15*, *16*). We have now discovered that DMSO is not the right choice of solvent to dissolve anthocyanins and betanins for the assay. The rapid color change of these compounds in solution indicated that the chemical integrity of both betanin and anthocyanins was

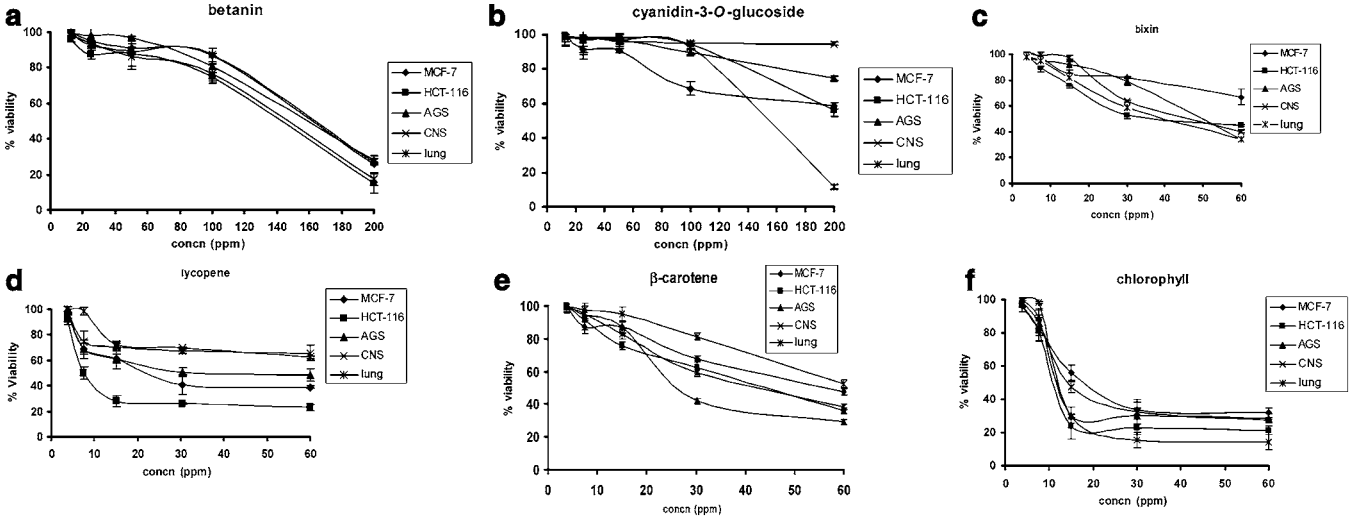

Figure 4. Tumor cell growth inhibitory activities of (**a**) betanin and (**b**) cyanidin-3-*O*-glucoside at 100 μg/mL, (**c**) lycopene, (**d**) bixin, (**e**) β-carotene, and (**f**) chlorophyll at 50 μg/mL against human cancer cell lines MCF-7 (breast), HCT-116 (colon), AGS (stomach), SF-268 (CNS), and NCI-H460 (lung). The results are for the average of three independent experiments conducted in triplicate. The vertical bars represent the standard deviation of three individual experiments conducted in triplicate.

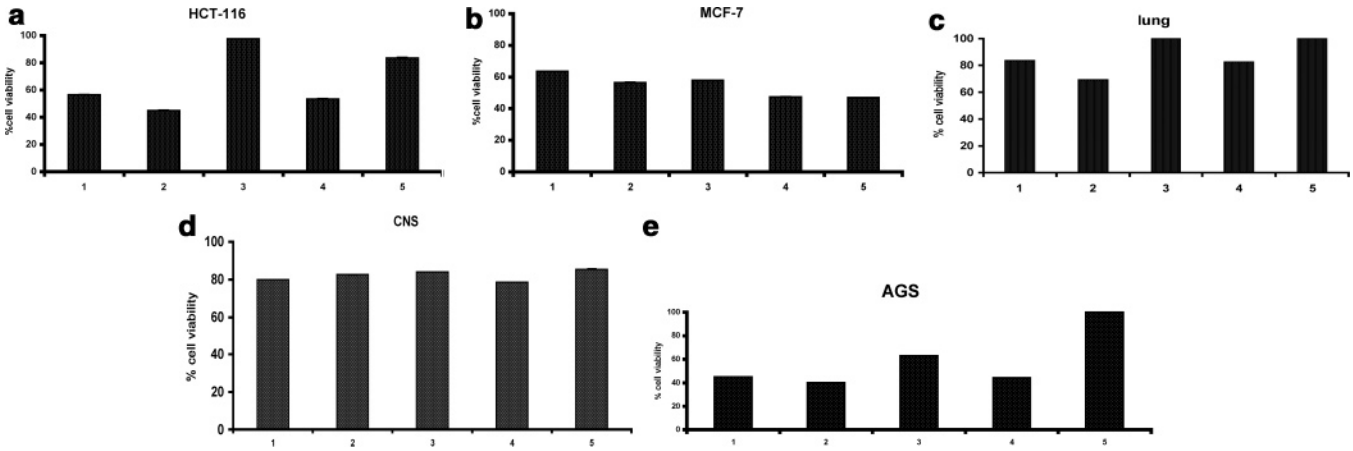

Figure 5. Synergistic effects of pigment combinations 1–5 on human tumor cell lines: (**a**) colon, (**b**) breast, (**c**) lung, (**d**) CNS, and (**e**) stomach. Betanin, anthocyanin, bixin, lycopene, β-carotene, and chlorophyll were assayed at 150, 150, 40, 10, 30, and 10 μg/mL, respectively. The combinations of pigments assayed were (1) bixin + β-carotene + lycopene, (2) bixin + β-carotene + lycopene + chlorophyll, (3) bixin + β-carotene + lycopene + betanin + cyanidin-3-*O*-glucoside, (4) bixin + β-carotene + lycopene + chlorophyll + betanin + cyanidin-3-*O*-glucoside, and (5) betanin + cyanidin-3-*O*-glucoside. The results are for an average of three independent experiments conducted in triplicate. The vertical bars represent the standard deviation of three individual experiments conducted in triplicate.

compromised by DMSO. Both of these water soluble pigments were affected irreversibly by the DMSO solvent interaction.

Because our earlier reports on the tumor cell growth inhibitory effects of anthocyanins were for DMSO solutions, in this study, we have assayed cyanidin-3-*O*-glucoside for cell proliferation inhibitory activity using water solutions. The results indicated that it is highly effective against lung cancer proliferation (**Figure 4b**). Similarly, betanin showed potent inhibition of cell growth at 200 ppm against all cell lines tested. However, the mixture of betanin and anthocyanin (combination 5, **Figure 5a**–**e**) reduced their respective potency considerably. Our results indicated interaction between betacyanin and anthocyanin and caused a reduction in their respective potency.

The carotenoids are reported to be excellent antioxidants. They protect oxidative damage to DNA, and protein and lipids are thought to be primarily responsible for the inhibition of cell proliferation (*18*). Bixin was reported to bind to protein and reduce cholesterol, triacylglycerols, and blood glucose levels (*19*), whereas β-carotene was reported to be effective against several types of cancers (*20*). Also, β-carotene was detected in the human plasma bound to low-density lipoprotein and it acted as an antioxidant (*21*). The leafy vegetables are reported to reduce the mutagenic activity (*22*), and a chlorophyll-rich diet has been implicated in the prevention or partial delay of the growth of certain types of tumors (*23*). Lycopene and β-carotene exhibited strong lipid peroxidation and COX enzymes inhibitory activities in the present study. However, bixin was not very effective in inhibiting COX enzymes. All three carotenoids tested were nonselective in inhibiting the COX enzymes. Although not selective, chlorophyll showed good COX inhibitory activity. It is interesting to note that the water soluble pigments, betanin and anthocyanins, showed less potency than the lipid soluble pigments lycopene, β-carotene, and chlorophyll in COX enzyme inhibitory assay. Even though bixin belonged to the carotenoid group, it was less efficacious than lycopene and β-carotene. This is the first report of lipid peroxidation and COX enzyme inhibitory activities of these pigments.

Among all of the natural colors tested, the lipid soluble pigments showed higher antioxidant, antiinflammatory, and anticancer activities than hydrophilic pigments. However, at

higher concentrations, the water soluble pigments exhibited an activity similar to lipid soluble pigments. The synergistic studies revealed that a combination of betanin and anthocyanin reduced their respective biological activities. Therefore, a food or supplement containing these two pigments may not contribute to its expected health benefits. Also, a combination of lycopene, β-carotene, and bixin showed a negative impact on their biological activities. However, the combination of chlorophyll and lycopene showed a strong inhibition of tumor cell proliferation against all of the cell lines studied. The inhibition of lipid peroxidation, cyclooxygenase enzymes, and tumor cell proliferation by these natural pigments is significant. An adequate dosage of these pigments as part of the regular diet could improve health and quality of life. However, the question that remains to be answered is whether these pigments in the right dose interact or provide the expected potency in vivo when consumed alone and in combination.

ACKNOWLEDGMENT

This is a contribution from the Michigan State University Agricultural Experiment Station. We thank Dr. Dave Dewitt, Department of Biochemistry and Molecular Biology at Michigan State University, for supplying cloned insect cells to isolate COX-2 enzyme.

LITERATURE CITED

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Received for review June 13, 2005. Revised manuscript received September 9, 2005. Accepted September 14, 2005.

JF051399J