JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant and Antiproliferative Activities of Raspberries

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Raspberries are rich in phenolic phytochemicals. To study the health benefits of raspberries, four fresh raspberry varieties (Heritage, Kiwigold, Goldie, and Anne) were evaluated for total antioxidant and antiproliferative activities. The total amount of phenolics and flavonoids for each of the four raspberry varieties was determined. The Heritage raspberry variety had the highest total phenolic content (512.7 ± 4.7 mg/100 g of raspberry) of the varieties measured followed by Kiwigold (451.1 ± 4.5 mg/100 g of raspberry), Goldie (427.5 ± 7.5 mg/100 g of raspberry), and Anne (359.2 ± 3.4 mg/100 g of raspberry). Similarly, the Heritage raspberry variety contained the highest total flavonoids (103.4 ± 2.0 mg/100 g of raspberry) of the varieties tested, followed by Kiwigold (87.3 ± 1.8 mg/100 g of raspberry), Goldie (84.2 ± 1.8 mg/100 g of raspberry), and Anne (63.5 ± 0.7 mg/100 g of raspberry). The color of the raspberry juice correlated well to the total phenolic, flavonoid, and anthocyanin contents of the raspberry. Heritage had the highest *a*/*b* ratio and the darkest colored juice, and the Anne variety showed the lowest phytochemical content and the palest color. Heritage raspberry variety had the highest total antioxidant activity, followed by Kiwigold and Goldie, and the Anne raspberry variety had the lowest antioxidant activity of the varieties tested. The proliferation of $HepG_2$ human liver cancer cells was significantly inhibited in a dose-dependent manner after exposure to the raspberry extracts. The extract equivalent to 50 mg of Goldie, Heritage, and Kiwigold fruit inhibited the proliferation of those cells by 89.4 ± 0.1, 88 ± 0.2, and 87.6 ± 1.0%, respectively. Anne had the lowest antiproliferative activity of the varieties measured but still exhibited a significant inhibition of 70.3± 1.2% with an extract equivalent to 50 mg of fruit. The antioxidant activity of the raspberry was directly related to the total amount of phenolics and flavonoids found in the raspberry ($p < 0.01$). No relationship was found between antiproliferative activity and the total amount of phenolics/flavonoids found in the same raspberry ($p > 0.05$).

KEYWORDS: Antioxidant; cancer; cell culture; antiproliferative activity; phenolics; flavonoids; raspberry; fruit

INTRODUCTION

There is an abundance of evidence that regular consumption of fruits and vegetables is associated with reduced risks of chronic diseases, such as cancers and cardiovascular disease (*1*, *2*). Fruits and vegetables are a primary food source providing essential nutrients for sustaining life; they also contain a variety of phytochemicals such as phenolics and flavonoids, which provide important health benefits (*2*−*4*). Free radical induced oxidative stress has been associated with several cellular toxic processes including oxidation damage to protein and DNA, membrane lipid oxidation, enzyme inactivation, and gene mutation that may lead to carcinogenesis (*5*). Intake of sufficient amounts of antioxidants is necessary to prevent free radical induced oxidative stress. It was reported that the majority of the antioxidant capacity of fruits and vegetables may come from total phenolics and flavonoids, because the antioxidant capacity of the vitamin C found in apple with skin accounts for only 0.4% of the total antioxidant activity (*6*).

A variety of dietary plant phenols and flavonoids have been found to have anticancer activity. Quercetin has been shown to inhibit the proliferation of azoxymethanol-induced colonic epithelial tumor cells in mice (*7*). Also, a phytochemical extract of apple inhibited colon cancer and liver cancer cell growth in

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10.1021/jf0111209 CCC: $22.00
Published on Web 04/04/2002

a dose-dependent manner (*6*). Raspberries are high in phenolic phytochemicals, particularly flavonoids and anthocyanin pigments, which gives these raspberries their characteristic color (*8*). This experiment was designed to (1) measure the total antioxidant activity of raspberries by the total oxyradical scavenging capacity (TOSC) assay and relate this to the total phenolic and flavonoid contents and (2) determine the ability of raspberry extracts to inhibit human liver cancer cell growth in vitro.

MATERIALS AND METHODS

Chemicals. Glucagon, insulin, hydrocortisone, and α-keto-γ-methiolbutyric acid (KMBA) were purchased from the Sigma Chemical Co. (St. Louis, MO), and 2,2′-azobis(amidinopropane) (ABAP) was obtained from Wako Chemicals (Richmond, VA).

Raspberry Preparation and Storage. Fresh Heritage, Kiwigold, Goldie, and Anne raspberry varieties were harvested at ripe stage when easily separated from the receptacle from the New York Agriculture Experiment Station orchards (Geneva, NY). Fresh raspberries were extracted using 80% acetone as reported previously in our laboratory (*6*). The extracts were stored at −70 °C until used.

Optical Sensor Rating: *a*/*b* Ratio. For each raspberry variety, 2−100 g samples of fruit were manually pressed through four layers of cheesecloth to obtain 25 mL of juice for each sample. Each sample was measured with a D25 L optical sensor (Hunter Lab, Reston, VA) for the values of *L*, *a*, and *b*. The two readings were then averaged, and a ratio of *a*/*b* was calculated. Moore found that the ratio of *a*/*b* was the measurement most highly correlated to the total anthocyanin content in raspberry fruit (*9*).

Determination of Total Phenolic and Flavonoid Contents. The total phenolic content of the four varieties of raspberries was determined using a modified Folin−Ciocalteu colorimetric method (*6*, *10*, *11*). Results are expressed as milligrams of gallic acid equivalents. Total flavonoid content was determined by a colorimetric method described previously (*6*, *11*, *12*). Briefly, 0.25 mL of the phytochemical extract was diluted with 1.25 mL of distilled water. Then 75 μL of a 5% $NaNO_2$ solution was added to the mixture. After 6 min, 150 μL of a 10% $AlCl_3 \cdot 6H_2O$ solution was added, and the mixture was allowed to stand for another 5 min. Half of a milliliter of 1 M NaOH was added, and the total was made up to 2.5 mL with distilled water. The solution was well mixed, and the absorbance was measured immediately against the prepared blank at 510 nm using an MRX II DYNEX Technologies spectrophotometer in comparison with the standards prepared similarly with known (+)-catechin concentrations. The results are expressed as milligrams of catechin equivalents. Data are reported as means ± SD for at least three replications.

Determination of Anthocyanin Content. The anthocyanin content of raspberries was determined using a modified pH differential method described previously (*13*−*15*). Briefly, 20 g of fruit was blended with 40 mL of extracting solvent (95% ethanol/1.5 N HCl, 85:15). The extract was collected through filtration with an additional 30 mL of solvent washing. The residue was soaked with 70 mL of extracting solvent, and the extract was collected after 2 h. The total extracts were pooled and brought up to 200 mL. The pH was adjusted to 1.0 ± 0.01. Absorbance readings were taken at 533 nm on a BioMate 3 series spectrophotometer (Thermo Spectronic, Rochester, NY). Absorbance readings for the undiluted extracts using a 1 cm cuvette at 533 nm were converted to total milligrams of cyanidin 3-galactoside using the following formula: $(A_{533} - A_{700}) \times 200 \times 5/98.2$. Heritage extract was diluted by a factor of 10. Data are reported as means ± SD for at least three replications.

Quantification of the Total Antioxidant Activity. The total antioxidant activity of the phytochemical extracts from raspberry cultivars was measured by using the total oxyradical scavenging capacity (TOSC) assay (*6*, *16*). Antioxidant activity was assessed at four different time points (15, 30, 45, and 60 min) and six different concentrations to determine the TOSC value. The TOSC value of each sample concentration was calculated using the integration of the area under the kinetics curve. The TOSC value was quantified according to the following equation:

$$\mathrm{TOSC} = 100 - \left(\int \mathrm{SA} / \int \mathrm{CA} \times 100\right)$$

$\int$SA and $\int$CA are the integrated areas from the sample and control reaction, respectively. The median effective dose (EC_{50}) of TOSC is expressed as micrograms of fruit per milliliter of reaction buffer for all samples calculated. All values of EC_{50} presented are means ± SD for at least three replications.

Measurement of Cell Proliferation. $HepG_2$ cells (The American Type Culture Collection, ATCC, Rockville, MD) were maintained in Williams medium E (WME), containing 10 mM Hepes, 5 μg/mL insulin, 2 μg/mL glucagon, 0.05 μg/mL hydrocortisone, and 5% fetal bovine serum (Gibco, Life Technologies, Grand Island, NY). $HepG_2$ cells were maintained at 37 °C in 5% CO_2 in an incubator. Cell concentrations of 2.5×10^4/mL in the growth media were placed in each well of a 96-well flat-bottom plate. The cell number was determined from a linear response curve during 96 h of cell growth. After 4 h of incubation at 37 °C in 5% CO_2, the growth medium was removed and media containing various concentrations (1, 5, 10, 20, 30, 40, and 50 mg/mL) of raspberry extracts were added to the cells. Control cultures received the extraction solution minus the raspberry extract and blank wells contain 100 μL of growth medium with no cells. After 96 h of incubation, cell proliferation was determined using the colorimetric MTS assay (MTS-based cell titer 96 nonradioactivity cell proliferation assay) (Promega, Madison, WI), a colorimetric method utilizing a tetrazolium reagent. Cell proliferation (percent) was determined at 96 h from the MTS absorbance (490 nm) reading for each concentration compared to the control. At least three replications for each sample were used to determine the cell proliferation (percent) value.

Table 1. Comparison of the Color (Optical Sensor *a*/*b*) of the Four Raspberry Varieties and the Phenolic, Flavonoid, and Anthocyanin Contents (Milligrams per 100 g of Fruit)

raspberry variety	color	total phenolics	total flavonoids	antho-cyanin	optical sensor *a*/*b*
Heritage	dark red	512.7 ± 4.7	103.4 ± 2.0	57.60 ± 0.76	4.92
Kiwigold	pink red	451.1 ± 4.5	87.3 ± 1.8	2.56 ± 0.03	2.84
Goldie	pink red	427.5 ± 7.5	84.2 ± 1.8	4.56 ± 0.1	2.24
Anne	yellow	359.2 ± 3.4	63.5 ± 0.7	0.17 ± 0.02	0.18

RESULTS

Phytochemicals and Total Antioxidant Activity of Four Varieties of Raspberries. Total phenolic and flavonoid contents of four varieties of raspberries are shown in **Table 1**. Heritage raspberry had the highest total phenolic content (512.7 ± 4.7 mg/100 g of fruit) of the varieties measured followed by Kiwigold (451.1 ± 4.5 mg/100 g of fruit) and then Goldie (427.5 ± 7.5 mg/100 g of fruit). Anne raspberry had the lowest total phenolic content (359.2 ± 3.4 mg/100 g of fruit). Heritage raspberry contained the highest total flavonoids (103.4 ± 2.0 mg/100 g of fruit) of the varieties tested, followed by Kiwigold (87.3 ± 1.8 mg/100 g of fruit) and Goldie (84.2 ± 1.8 mg/100 g of fruit). Anne raspberry also had the lowest content of flavonoids (63.5 ± 0.7 mg/100 g of fruit) of the varieties tested.

Heritage raspberry contained the highest anthocyanin content (57.6 ± 0.76 mg/100 g of fruit) of the varieties tested (**Table 1**), followed by Goldie (4.56 ± 0.10 mg/100 g of fruit) and Kiwigold (2.56 ± 0.03 mg/100 g of fruit). Anne raspberry also had the lowest content of anthocyanin (0.17 ± 0.02 mg/100 g of fruit) of the varieties tested.

The optical sensor ratings suggest that the color of the raspberry juice (**Figure 1**) correlated well to the total phenolic,

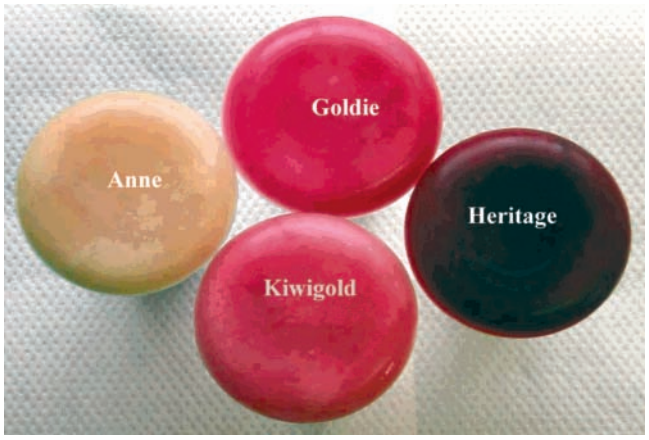

Figure 1. Color differences among the four raspberry cultivars.

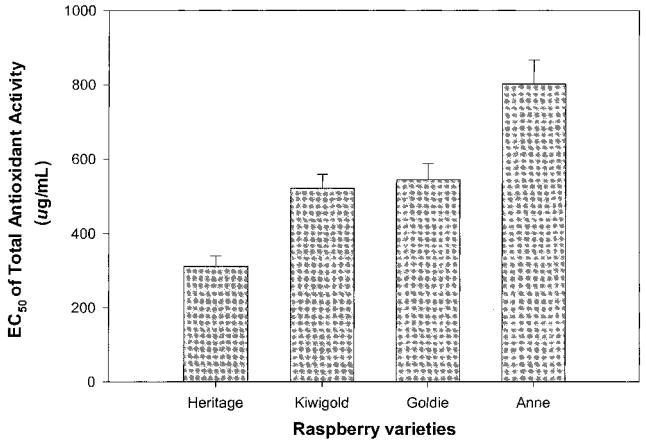

Figure 2. EC_{50} of total antioxidant activity for the four varieties of raspberries tested.

flavonoid, and anthocyanin contents of the raspberries (**Table 1**). Heritage had the highest *a*/*b* ratio and the darkest colored juice; Kiwigold and Goldie were similar in chemical phenolic/flavonoid contents and color, followed by the Anne variety, showing the lowest phytochemical content and the palest color.

The results of total antioxidant activity were expressed as the median effective dose (EC_{50}; **Figure 2**). A low EC_{50} translates to a higher total antioxidant activity. The Heritage raspberry had the lowest EC_{50} of total antioxidant activity (312.1 ± 28.3 μg/mL) of the varieties measured, followed by Kiwigold (521.7 ± 36.9 μg/mL) and Goldie (544.5 ± 12.3 μg/mL). Anne raspberry variety had the highest EC_{50} (801.8 ± 64.9 μg/mL). Because of the data obtained, it can be interpreted that the Heritage raspberry variety had the highest total antioxidant activity and the Anne raspberry variety the lowest antioxidant activity of the varieties tested.

Cell Proliferation. Cell proliferation was analyzed at 96 h after $HepG_2$ cells had been cultured with an extract equivalent to 0, 1, 5, 10, 20, 30, 40, or 50 mg/mL of raspberry fruit in the media using the MTS assay. $HepG_2$ cell proliferation was inhibited in a dose-dependent manner after exposure to the raspberry extracts. All varieties tested showed obvious antiproliferative activity. Goldie, Heritage, and Kiwigold showed similar antiproliferative activities at 50 mg/mL (89.4 ± 0.1, 88 ± 0.2, and 87.6 ± 1.0%, respectively), and their antiproliferative activities were higher than that of Anne. Anne had the lowest antiproliferative activity at 50 mg/mL (70.3 ± 1.2%) of the varieties measured. There is no significant difference ($p > 0.05$) between Goldie, Heritage, and Kiwigold in inhibition. **Figure 4** shows the EC_{50} of antiproliferative activity by different

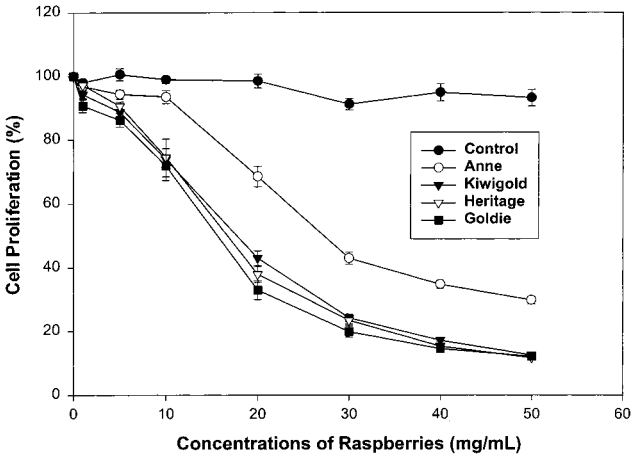

Figure 3. Percent inhibition of $HepG_2$ cell proliferation by raspberry extracts of the four varieties tested.

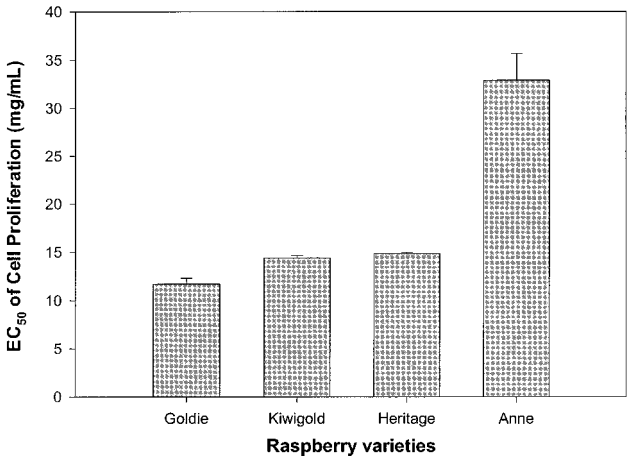

Figure 4. EC_{50} of antiproliferative activity by the extracts of the four raspberry varieties.

concentrations of the four raspberry varieties. Goldie raspberry had the lowest EC_{50} of antiproliferative activity (11.7 ± 0.6 mg/mL) of the varieties tested, but no significant difference ($p > 0.05$) was seen between Kiwigold (14.4 ± 0.3 mg/mL) and Heritage (14.8 ± 0.1 mg/mL). Anne raspberry, having the highest EC_{50} of antiproliferative activity (32.9 ± 0.8 mg/mL), was significantly different ($p < 0.01$) from the other three varieties. Anne raspberry had the lowest antiproliferative activity of the four varieties of raspberries tested.

Relationship between Phytochemical Contents, Total Antioxidant Activity, and Median Effective Dose (EC_{50}) of Inhibition of $HepG_2$ Cell Proliferation. There was an inverse ($R^2 = 0.988$) relationship between total phenolic content and the EC_{50} of total antioxidant activity of raspberries ($p < 0.01$; **Figure 5A)**. The higher phenolic concentrations resulted in a greater antioxidant activity. Similarly, there was an inverse ($R^2 = 0.996$) relationship between the EC_{50} of total antioxidant activity and flavonoid content of the raspberries ($p < 0.01$; **Figure 5B**). The higher the phenolic and flavonoid contents, the higher the antioxidant activity.

The relationships between total phenolic/flavonoid contents and median effective dose (EC_{50}) of the inhibition of $HepG_2$ cell proliferation were studied. There was no obvious relationship between total phenolics and inhibition of $HepG_2$ cell proliferation ($R^2 = 0.563$, $p > 0.05$). The relationship between total flavonoids and the inhibition of $HepG_2$ cell proliferation was not observed ($R^2 = 0.654$, $p > 0.05$). The total antioxidant

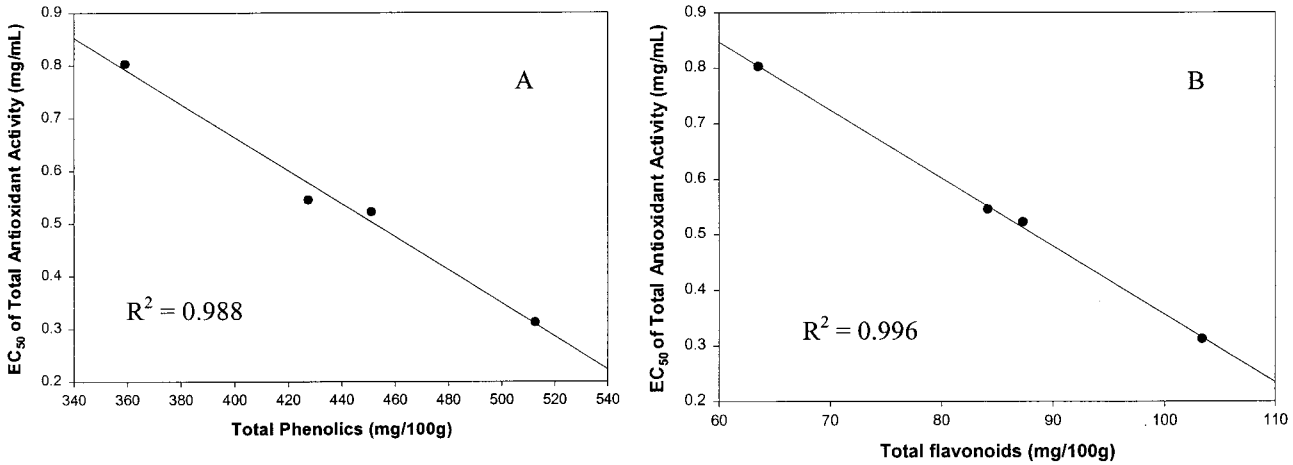

Figure 5. Relationship between total phenolic content (A), total flavonoid content (B), and EC_{50} of total antioxidant activity of four raspberry varieties.

activity was not correlated with inhibition of $HepG_2$ cell proliferation ($R^2 = 0.415$, $p > 0.7$).

DISCUSSION

Fruits and vegetables are good sources of antioxidants (*3*, *4*, *17*). Previously, most investigators focused mainly on the vitamin C, vitamin E, and β-carotene contents of fruits. However, the major components of fruit that act as antioxidants are phytochemicals such as phenolic compounds, flavonoids, and anthocyanins (*18*). The vitamin C in apples with skin accounts for only 0.4% of the total antioxidant activity (*6*), indicating that almost all of the antioxidant activity is from other phytochemicals in the fruit. Red and black raspberries are especially high in phenolic compounds compared to other lighter colored fruits and vegetables. The TOSC assay was used to determine the antioxidant activity of four varieties of raspberries, and this value was compared to the amounts of total phenolics and flavonoids determined in each of the varieties. The yellow fruited variety, Anne, has been found to contain low levels of phenolics, flavonoids, anthocyanin, and antioxidant activity. Heritage has dark red fruit, containing the highest levels of phenolics, flavonoids, anthocyanin, and antioxidant activity of the varieties tested. The pink-red fruited varieties Kiwigold and Goldie are sports of the Heritage variety. They display mutations in the anthocyanin biosynthesis pathway that reduce their pigment content. Both Kiwigold and Goldie are genetically identical to Heritage except for their anthocyanin content. The results show that Kiwigold and Goldie have higher antioxidant activity, total phenolics, and total flavonoids than Anne but less than Heritage. These data show that higher phenolic, flavonoid, and anthocyanin contents in the raspberry fruits contributes to their higher antioxidant activity. From these data it can be concluded that a darker colored raspberry has more antioxidant activity.

Raspberries contain ~25 mg/100 g vitamin C (*19*), a small portion of the antioxidant activity (1.42 μmol of vitamin C equivalents/g of raspberry). Therefore, the total antioxidant activity of raspberries was mainly from the other phytochemicals in the fruit rather than from the vitamin C. The antioxidant activity may be explained by looking at the combination of different phytochemicals functioning additively or synergistically accounting for the total antioxidant activity of raspberry.

Extracts of the four raspberry varieties were added to $HepG_2$ human liver cells to determine whether tumor cell proliferation could be inhibited. The results showed that all raspberry extracts tested at a concentration of $\geq$20 mg/mL had stronger inhibiting activity than the control ($p < 0.01$). Cell proliferation was inhibited in a dose-dependent manner when exposed to raspberry concentrations >10 mg/mL. The Anne variety of raspberry had a significantly lower inhibiting ability than the other three raspberry varieties ($p < 0.01$). It is worth pointing out that there was no significant difference ($p > 0.05$) between Kiwigold, Heritage, and Goldie in the inhibition of $HepG_2$ cell proliferation, although their antioxidant activities were different. Except for anthocyanin content, Kiwigold, Heritage, and Goldie are genetically identical. Pigment content is a factor affecting antioxidant activity but not a factor in the inhibition of cell proliferation. Therefore, it is assumed that phytochemicals other than anthocyanin in the raspberries were responsible for the inhibition of tumor cells. Additional experiments should be designed to identify the individual chemical compounds in the raspberry that inhibit proliferation of tumor cells.

The overall relationship between the total phenolics/flavonoids found in the raspberry varieties and the EC_{50} of antioxidant activity was statistically significant with a R^2 values of 0.988 ($p < 0.01$) and 0.996 ($p < 0.01$), respectively. This indicates that the total phenolic and flavonoid contents contribute significantly to the antioxidant activity of the raspberries tested. However, the relationship between the total phenolics/flavonoids and EC_{50} of $HepG_2$ cell inhibition was not significant ($R^2 = 0.563$, $p > 0.05$; and $R^2 = 0.654$, $p > 0.05$, respectively). Therefore, the inhibition of in vitro tumor cell proliferation by raspberries cannot be explained by the concentration of phenolic/flavonoid compounds alone. This suggests that other phytochemicals may play a major role in the antiproliferative activity of raspberries. This experiment suggests that the antioxidant activity was not interrelated with inhibition of $HepG_2$ cell proliferation ($R^2 = 0.415$, $p > 0.05$).

Endogenous oxidative DNA damage has been considered to be a significant factor in the initiation of human cancer. The cancer-protective effect of vegetables and fruits is attributed to the ability of the antioxidants in them to scavenge free radicals, preventing DNA damage and subsequent mutation. A trial in Linxian, China, demonstrated that the incidence of gastric cancer in groups who received a supplement of β-carotene, vitamin E, and selenium was much lower than that of the placebo group (*20*). It was suggested that antioxidants could prevent tumor initiation and act as a protective agent. With tumor progression, there are multiple mechanisms that are associated with the carcinogens. Therefore, the role of phytochemicals in regulating gene expression is worthy of investigation.

This study suggests that the phytochemicals in raspberries may have a significant effect on antioxidant and anticancer activities. It is clear that raspberries higher in phenolic and flavonoid compounds have higher antioxidant activity. The darker colored raspberry varieties showed greater antioxidant activity than the lighter colored variety, Anne. The antioxidant activity of the raspberry was directly related to the total amount of phenolics and flavonoids found in the raspberry, but there was no relationship between antiproliferative activity and the total amount of phenolics/flavonoids found in the same raspberry. The inhibition of cancer cell proliferation is attributed to some unknown compound(s) present in raspberry fruits. The additive and synergistic roles of phytochemicals may contribute significantly to the potent antioxidant activity and the ability to inhibit in vitro tumor cell proliferation.

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Received for review August 16, 2001. Revised manuscript received February 27, 2002. Accepted February 27, 2002.

JF0111209