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Antioxidant and Antiproliferative Activities of Strawberries

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Strawberries contain high levels of antioxidants, which have been correlated with a decreased risk of chronic disease. To more fully characterize the antioxidant profiles and possible associated health benefits of this fruit, the total free and bound phenolic, total flavonoid, and total anthocyanin contents of eight strawberry cultivars (Earliglow, Annapolis, Evangeline, Allstar, Sable, Sparkle, Jewel, and Mesabi) were measured. Cultivar effects on phenolic contents were compared with antioxidant capacities, as measured by the total oxyradical scavenging capacity (TOSC) assay, and to antiproliferative activities, as measured by inhibition of $HepG_2$ human liver cancer cell proliferation in vitro. Free phenolic contents differed by 65% between the highest (Earliglow) and the lowest (Allstar) ranked cultivars. The water soluble bound and ethyl acetate soluble bound phenolic contents averaged 5% of the total phenolic content of the cultivars. The total flavonoid content of Annapolis was 2-fold higher than that of Allstar, which had the lowest content. The anthocyanin content of the highest ranked cultivar, Evangeline, was more than double that of the lowest ranked cultivar, Allstar. Overall, free phenolic content was weakly correlated with total antioxidant activity, and flavonoid and anthocyanin content did not correlate with total antioxidant activity. The proliferation of $HepG_2$ human liver cancer cells was significantly inhibited in a dose-dependent manner after exposure to all strawberry cultivar extracts, with Earliglow exhibiting the highest antiproliferative activity and Annapolis exhibiting the lowest. No relationship was found between antiproliferative activity and antioxidant content.

KEYWORDS: Phytochenicals; phenolics; flavonoids; anthocyanins; antioxidant; antiproliferation; cancer; strawberry; *Fragaria* x *ananassa* Duch.

INTRODUCTION

The leading causes of mortality in the United States are heart disease and cancer (*1*). Dietary patterns are thought to influence the onset and progression of these and other chronic and degenerative diseases (*2*). Djuric et al. (*3*) showed that a diet high in fruits reduced oxidative damage to DNA, thus preventing a critical step in the onset of carcinogenesis. Data from the Nurses' Health Study and the Health Professionals' Follow-Up Study correlated each dietary daily serving of fruits or vegetables to a 4% risk reduction for coronary heart disease (*4*). The Caerphilly Study comparing Welsh men in the highest quintile of fruit intake with those in the lowest quintile observed a significantly reduced risk for all-cause cancer mortality for those in the highest quintile (*5*).

Numerous studies have suggested that the phytochemical content and corresponding antioxidant activity of fruits and vegetables contribute to their protective effect against chronic and degenerative diseases (*6*, *7*). Phytochemicals that exhibit antioxidative activity include phenolic compounds such as flavonoids, nitrogenous compounds such as chlorophyll derivatives, as well as tocopherols, carotenoids, and ascorbic acids. These natural antioxidants act by scavenging free radical species or by inhibiting the generation of reactive species during the course of normal cell metabolism, thus preventing damage to lipids, proteins, and nucleic acids and eventual cellular damage and death (*6*, *8*). Additionally, the antioxidants present in fruits and vegetables have been found to exhibit anticarcinogenic and antimutagenic activity (*9*); individual plant phenolics appear to exhibit different anticarcinogenic mechanisms including inactivating potentially carcinogenic reactive electrophiles and scavenging reactive oxygen species that may initiate tumor production. Much information is available on the reported inhibitory effects of specific plant phenolic compounds and extracts on mutagenesis and carcinogenesis and the role of antioxidant activity on these effects (*10*, *11*).

Several studies have shown that the strawberry generally possesses a high level of antioxidant activity, which is linked to the levels of phenolic compounds in the fruit (*6*, *12*, *13*). Wang and Jiao (*14*) showed that strawberry juice extracts

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exhibited a high level of antioxidant capacity against free radical species including superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen; the percent inhibition of each active oxygen species varied among the six strawberry cultivars studied. Flavonoids, major phenolic compounds in strawberries, have been shown to exhibit antioxidant (*15*, *16*) and anticancer properties (*17*). The contents of the flavonoid groups, flavonols, and anthocyanins in strawberries have been associated indirectly and directly, respectively, with the total antioxidant capacity for low-density lipoproteins of the fruit extracts (*6*). Anthocyanins are typically present at high levels in strawberries and are thought to significantly contribute to the total antioxidative activity of this fruit (*18*). Total flavonoid content, as well as total anthocyanin content, can vary among cultivars, and these may affect the antioxidant and overall protective benefits of individual total phenolic compounds and are worth further investigation.

Strawberry extracts have been found to have higher antioxidant activity, as indicated by the oxygen radical absorbance capacity assay, than extracts from plum, orange, red grape, kiwifruit, pink grapefruit, white grape, banana, apple, tomato, pear, and honeydew melon (*16*). Using a total antioxidant oxyradical scavenging assay (TOSC), however, Sun et al. (*13*) identified a different ranking within a slightly different variety of fruit types. Strawberry extracts had higher antioxidant activity than extracts from peach, lemon, banana, pear, orange, grapefruit, and pineapple, but cranberry, apple, and grape extracts exhibited greater antioxidant activity than strawberry extracts. These differences may have been due to the methods used to measure antioxidant activity or to source, condition, and/or identity of the particular cultivars tested. Several strawberry cultivars have been found to display significantly higher levels of antioxidant activity than others (*19*), and the individual flavonoid and phenolic acid compounds have been found to differ among cultivars, as determined by high-performance liquid chromatography (HPLC) analysis (*20*). These differences may impact the ultimate overall antioxidative and anticarcinogenic activity. Greater knowledge of the antioxidant activity of strawberry cultivars may influence their market value; strawberries ranked fourth in cash receipts for farmers, totaling $1.1 billion in 1999 (*21*). Thus, it is of interest to further explore the differences in phenolic content and antioxidant activity between different strawberry cultivars in order to provide a more complete characterization of their benefits.

Previous studies have focused mainly on the free phenolic content and antioxidant activity of strawberries. However, antioxidants can exist in both free and bound forms. Recent research in our laboratory suggests that bound antioxidants in fruits and vegetables have been overlooked and total antioxidant activity has therefore been underestimated (*13*, *22*). This experiment was designed to additionally expound upon the research performed previously on strawberries and provide a more complete phenolic profile for several cultivars. The objectives were to (i) determine the content of both free and bound phenolics in eight strawberry cultivars; (ii) determine the total flavonoid and total anthocyanin contents; (iii) measure the total antioxidant activity; (iv) determine the antiproliferative activity of strawberry extracts on human liver cancer cell growth in vitro; and (v) identify correlations between total phenolic, flavonoid, and anthocyanin contents and antioxidant and/or antiproliferative activities.

MATERIALS AND METHODS

Chemicals. Sodium nitrite, (+)-catechin, Folin−Ciocalteu (FC) reagent, hydrochloric acid, glucagon, hydrocortisone, insulin, and α-keto-γ-methiolbutyric acid were purchased from Sigma Chemical Co. (St. Louis, MO). Aluminum chloride, sodium hydroxide, methanol, and acetone were purchased from Fisher Scientific (Pittsburgh, PA). Gallic acid was purchased from ICN Biomedical Inc. (Costa Mesa, CA). 2,2′-Azobis(amidinopropane) was purchased from Wako Chemicals (Richmond, VA).

Plant Material. Annapolis, Allstar, Earliglow, Evangeline, Mesabi, Sable, Sparkle, and Jewel strawberries were harvested at the light ripe red stage of ripening from the Cornell Orchards (Ithaca, NY). The planting was 3 years old, and the plants were grown and managed using standard procedures for the northeastern United States (*23*). Berries were selected that were free from visible blemish or disease.

Extraction of Free and Bound Phenolic Compounds. Free phenolics were extracted from fresh strawberries using 80% acetone (*24*). Bound phenolics were extracted from the remaining residue using the method of Chu et al. (*22*) The residue was hydrolyzed for 1 h with 20 mL of 2 N sodium hydroxide. The mixture was neutralized with hydrochloric acid and extracted six times with ethyl acetate. The ethyl acetate fraction was evaporated to dryness at 45 °C, and antioxidants recovered with distilled water were designated as Bound-E. Water soluble bound antioxidants were recovered by filtering through muffled Celite using a 20% methanol in ethyl acetate solution as the mobile phase. The water soluble fraction was evaporated to dryness at 45 °C, and recovered antioxidants were designated as Bound-W. All extracts were stored at −40 °C until use. All extractions were performed in triplicate.

Determination of Total Phenolic Content. The total phenolic content of the free, Bound-E, and Bound-W extracts was determined using the FC colorimetric method (*25*) as modified by Dewanto et al. (*26*). A 125 μL aliquot of the extract was mixed with 0.5 mL of distilled water and subsequently with 125 μL of FC reagent. After 6 min, 1.25 mL of a 7% aqueous sodium carbonate solution was added. Water was added to bring the total volume to 3 mL, and samples were allowed to stand for 90 min. The samples were then read at 760 nm vs a prepared blank with an MRX II DYNEX spectrophotometer (DYNEX Technologies, Inc., Chantilly, VA) and compared with a known concentration range of gallic acid standards similarly prepared. All results were expressed as milligrams of gallic acid equivalents per 100 g fresh weight of strawberry. Data were reported as a mean ± SD for three replications.

Determination of Total Flavonoid Content. The total flavonoid content of the acetone extract was determined using a modified colorimetric method described previously (*26*, *27*). A 250 μL aliquot of the extract was mixed with 1.25 mL of distilled water and subsequently with 75 μL of a 5% $NaNO_2$ solution. After 6 min, 150 μL of a 10% $AlCl_3 \cdot 6H_2O$ solution was added and allowed to stand for 5 min before the further addition of 0.5 mL of 1 M NaOH. Water was added to bring the total volume to 2.5 mL, and the samples were read immediately at 510 nm against a prepared blank using an MRX II DYNEX spectrophotometer (DYNEX Technologies, Inc.). All values were expressed as milligrams of catechin equivalents per 100 g fresh weight of strawberry. Data were reported as means ± SD for three replications.

Determination of Anthocyanin Content. The total anthocyanin content of the acetone extract was determined using a modified pH differential method described previously (*24*, *28*, *29*). A Beckman DU640B spectrophotometer was used to measure absorbance at 510 and 700 nm in buffers at pH 1.0 and 4.5. Absorbance readings were converted to total milligrams of cyanidin 3-glucoside per 100 g fresh weight of strawberry using the molar extinction coefficient of 26 900 and absorbance of $A = [(A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}]$. Data were reported as means ± SD for three replications.

Quantification of the Total Antioxidant Activity. The total antioxidant activity of the acetone extract was determined using the TOSC assay (*30*) as modified in our laboratory (*26*). Antioxidant activity was measured at four different time points (15, 30, 45, and 60 min) and four different extract concentrations (1.0, 2.5, 5.0, and 10.0 mg/mL) 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: $TOSC = 100 - (\int SA/\int CA \times 100)$, where $\int SA$

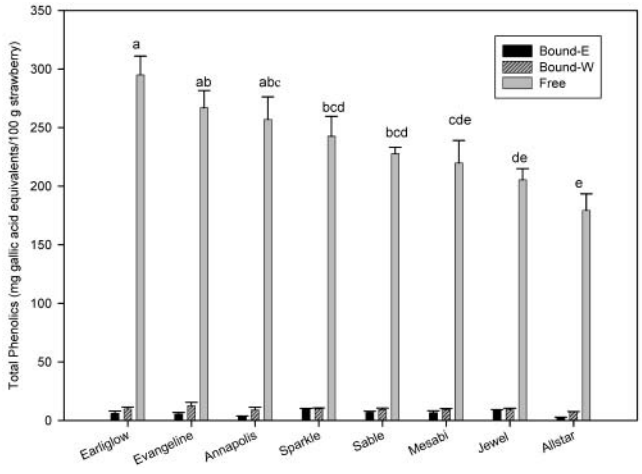

Figure 1. Free, Bound-E, and Bound-W phenolic content of the eight strawberry cultivars tested (mean ± SD, $n = 3$). Cultivars with no letters in common are significantly different ($p < 0.05$).

and ∫CA are the integrated areas from the sample and control reaction, respectively. The results were calculated as μmol of vitamin C equivalents per gram of strawberry and reported as means ± SD for three replications.

Measurement of Cell Proliferation. $HepG_2$ liver cancer cells (The American Type Culture Collection, ATCC, Rockville, MD) were maintained in Williams medium E, 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) at 37 °C in 5% CO_2 in an incubator (*31*, *32*). Antiproliferative activities of strawberry extracts were measured by the MTS-based cell titer 96 nonradioactive cell proliferation assay described previously (*24*). Aliquots of growth media cell concentrations of 2.5×10^4 cells/well 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, the growth medium was removed and media containing increasing concentrations (0, 5, 10, 20, 30, 40, 50, and 75 mg/mL) of strawberry extracts were added to the cells. Control cultures received the extraction solution minus the strawberry extract, and blank wells contained 100 μL of growth medium with no cells. After 96 h of incubation, cell proliferation was determined using the colorimetric MTS assay with a tetrazolium reagent. The MTS absorbance was read at 490 nm using an MRX II DYNEX spectrophotometer (DYNEX Technologies, Inc.). Data were reported as mean ± SD for three replications.

Statistical Analysis. Statistical analysis was performed using Minitab software (Minitab, Inc., State College, PA). Cultivar differences were determined using analysis of variance and Tukey's multiple comparison method with a family error rate of 0.05. Significance of relationships was determined using regression analysis of variance. All data were reported as mean ± standard deviation of three replications.

RESULTS

Free and Bound Phenolic Contents. The total free and bound phenolic contents of the eight strawberry cultivars are shown in **Figure 1**. Earliglow, Evangeline, and Annapolis had the highest free phenolic contents, averaging 273 mg gallic acid equivalents/100 g fruit, while the lowest content was measured in Mesabi, Jewel, and Allstar, averaging 202 mg gallic acid equivalents/100 g fruit. There was a 65% difference in free phenolic content between the highest and the lowest ranked cultivars, Earliglow and Allstar ($p < 0.05$). The Bound-E phenolic content of Sparkle (9.6 ± 0.6 mg gallic acid equivalents/100 g fruit) was higher ($p < 0.05$) than the contents of Evangeline, Annapolis, and Allstar (average of 3.5 mg gallic acid equivalents/100 g fruit). The only significant difference in

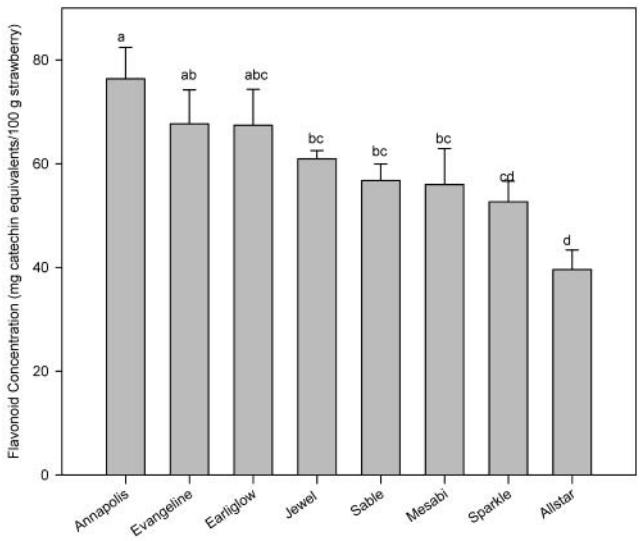

Figure 2. Flavonoid content of the eight strawberry cultivars tested (mean ± SD, $n = 3$). Cultivars with no letters in common are significantly different ($p < 0.05$).

Bound-W phenolic content was between Evangeline (12.6 mg gallic acid equivalents/100 g fruit) and Allstar (7.3 mg gallic acid equivalents/100 g fruit) strawberries ($p < 0.05$). Only weak relationships were detected between free phenolics and Bound-E phenolics ($R^2 = 0.005$, $p > 0.05$) or free phenolics and Bound-W phenolics ($R^2 = 0.431$, $p > 0.05$).

Total Flavonoid and Total Anthocyanin Contents. Annapolis, Evangeline, and Earliglow strawberries had the highest flavonoid content (averaging 70.5 mg catechin equivalents/100 g fruit), while Sparkle and Allstar had the lowest (46.2 mg catechin equivalents/100 g fruit; **Figure 2**).

Anthocyanin contents were similar in most cultivars, averaging 41.4 mg cyanidin 3-glucoside/100 g fruit (**Figure 3**). The lowest content was measured in Allstar (21.9 ± 1.6), but statistically, this cultivar was similar to Sable and Earliglow. There was approximately a 2-fold difference in both flavonoid and anthocyanin contents between the highest and the lowest ranked cultivars ($p < 0.05$), although the relative ranking of cultivars varied for each compound.

Total Antioxidant Activity. The results of the TOSC assay for total antioxidant activity were expressed as μmol of vitamin C equivalents per gram of strawberry (**Figure 4**). The total antioxidant activity of Earliglow (134.1 ± 3.0) was highest. The lowest total antioxidant activity occurred in Allstar, while activity in the other cultivars ranged from 59.9 μmol of vitamin C equivalents per g for Mesabi to 36.7 μmol of vitamin C equivalents per g for Jewel. There was a weak positive correlation between free phenolic content and total antioxidant activity of strawberries (**Figure 5**). However, associations were weak between flavonoid content and total antioxidant activity ($R^2 = 0.188$, $p > 0.05$) or anthocyanin content and total antioxidant activity ($R^2 = 0.426$, $p > 0.05$).

Inhibition of Cell Proliferation. Cell proliferation was analyzed at 96 h after incubation of $HepG_2$ cells with media containing extracts of 0, 5, 10, 20, 30, 40, 50, and 75 mg/mL of strawberry fruit extract using the MTS assay. $HepG_2$ cell proliferation was inhibited in a dose-dependent manner after exposure to all of the strawberry extracts (**Figure 6**). A 50 mg/mL concentration of strawberry extract inhibited cell proliferation: Earliglow (89.1 ± 0.9%), Evangeline (88.7 ± 0.7%), Sable (86.1 ± 5.0%), Jewel (83.2 ± 2.7%), Sparkle (78.5 ± 3.3%), Mesabi (76.4 ± 1.7%), Allstar (72.2 ± 2.2%), and Annapolis

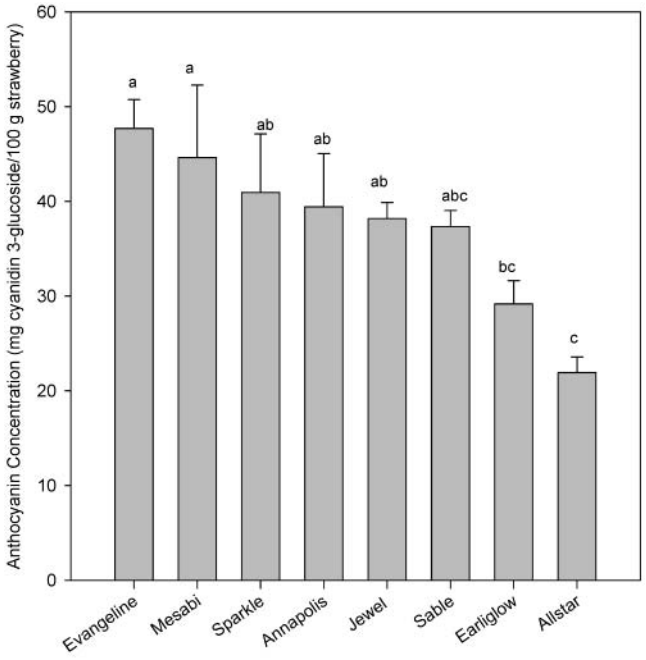

Figure 3. Anthocyanin content of the eight strawberry cultivars tested (mean ± SD, $n = 3$). Cultivars with no letters in common are significantly different ($p < 0.05$).

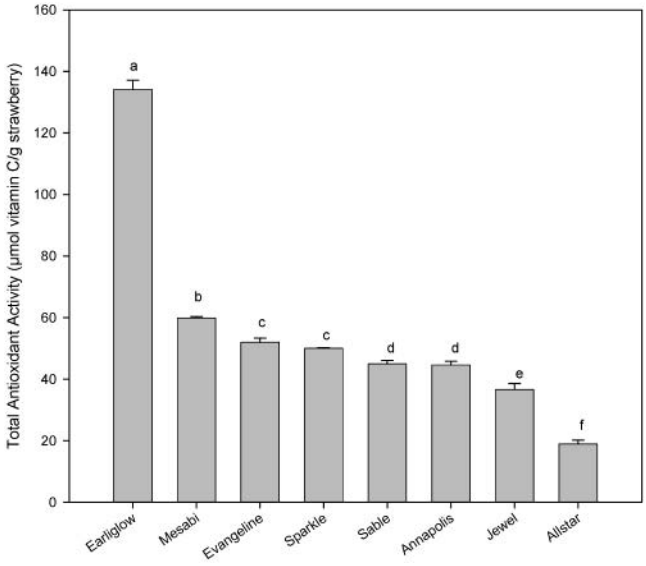

Figure 4. Total antioxidant activity of the eight strawberry cultivars tested (mean ± SD, $n = 3$). Cultivars with no letters in common are significantly different ($p < 0.05$).

(68.2 ± 0.0%). Antiproliferative activity was also measured as the median effective dose (EC_{50}), where a low EC_{50} value translates to a high antiproliferative value (**Figure 7**). The EC_{50} values for the eight cultivars were measured as follows: Earliglow (14.9 ± 0.5 mg/mL) followed by Evangeline (18.8 ± 0.9), Sable (22.3 ± 2.2), Sparkle (23.1 ± 2.5), Jewel (24.7 ± 1.3), Allstar (27.0 ± 5.1), Mesabi (28.0 ± 2.0), and Annapolis (31.6 ± 2.4). The EC_{50} of Earliglow was significantly lower than all other cultivars except Evangeline ($p < 0.05$). There was no obvious relationship between free phenolic content and inhibition of cell proliferation ($R^2 = 0.332$, $p > 0.05$) or flavonoid content and the inhibition of cell proliferation ($R^2 = 0.013$, $p > 0.05$). Additionally, there was no correlation between total antioxidant activity and inhibition of cell proliferation ($R^2 = 0.463$, $p > 0.05$).

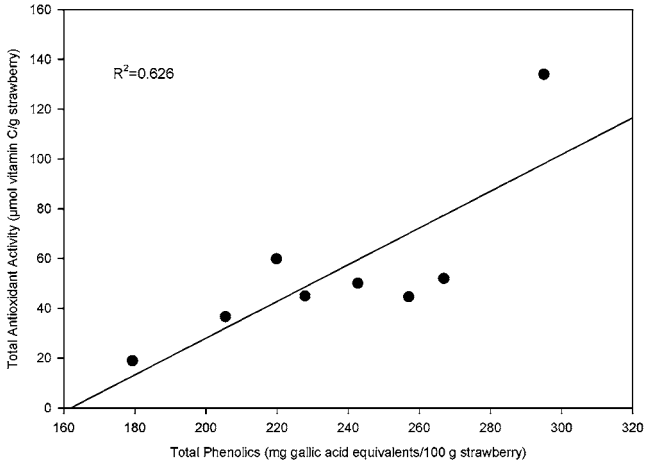

Figure 5. Relationship between total phenolic content and total antioxidant activity.

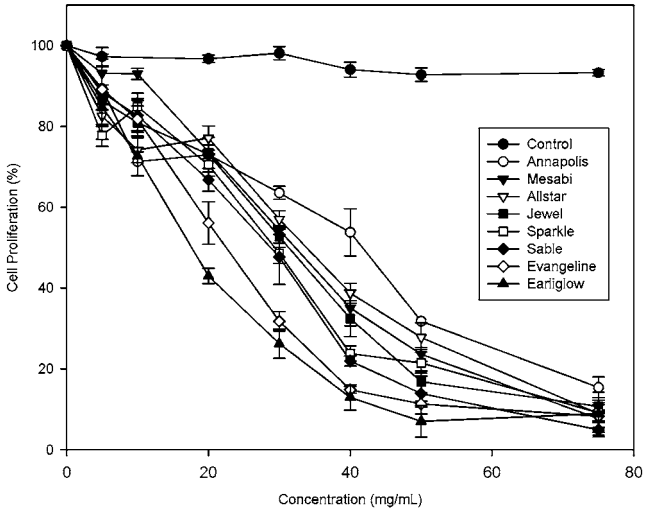

Figure 6. Percent inhibition of $HepG_2$ cell proliferation by strawberry extracts of the eight cultivars tested (mean ± SD, $n = 3$).

DISCUSSION

Strawberries are a good source of dietary antioxidants (*13*, *19*, *20*). The focus of dietary antioxidant research has primarily been on vitamin C, vitamin E, and β-carotene. However, recent research has found that the vitamin C content in whole apples contributes only 0.4% of the total antioxidant activity; the remaining antioxidant activity is derived largely from phytochemicals such as phenolics (*33*). A positive correlation between free phenolics and total antioxidant activity in a number of different fruits has been reported (*6*, *13*, *24*, *34*, *35*). Our study indicates that phenolics in strawberries account for a major portion of the total antioxidant activity of strawberries ($R^2 = 0.626$, $p < 0.05$).

Our study also confirms that significant differences in phytochemical content can exist among strawberry cultivars. A study (*19*) using strawberry cultivars grown in Maryland showed a similar pattern of phenolic content and total antioxidant activity to that quantified from the New York state-grown cultivars studied in this experiment. Only Earliglow and Allstar were common in both studies. Earliglow fruit extracts ranked higher than Allstar for both total phenolic content and antioxidant activity (*19*). Despite possible effects of cultivation site on photochemical contents (*20*), the data patterns and actual values for phytochemical content and antioxidant activity are similar between both studies.

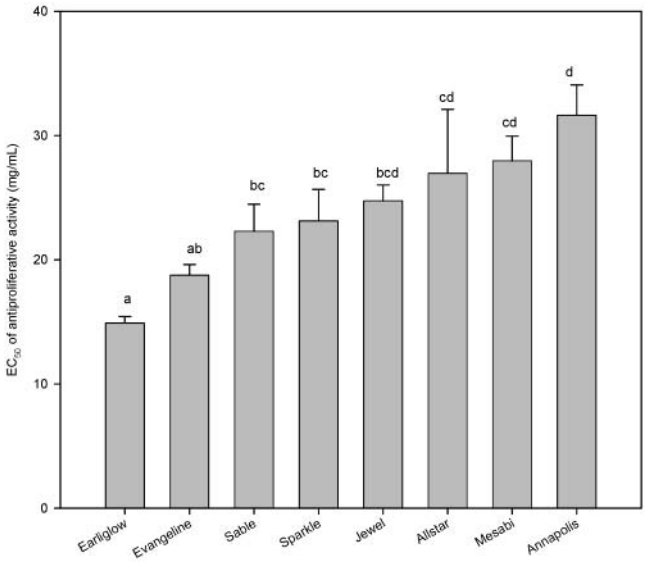

Figure 7. EC_{50} of antiproliferative activity by extracts of the eight strawberry cultivars (mean ± SD, $n = 3$). Cultivars with no letters in common are significantly different ($p < 0.05$).

Previous studies have focused on free phenolic content without inclusion of bound phenolic data. Our study demonstrates that bound phenolics in strawberries account for approximately 5% of the total phenolic content. Additionally, the cultivars showed a significant difference with respect to bound phenolics, with a 4-fold difference in Bound-E phenolics and a 2-fold difference in Bound-W phenolics between the lowest and the highest ranked cultivars. Bound phenolics have demonstrated stability during mechanical processing and thus may go undigested in the stomach and small intestine and ultimately exert bioactivity in the colon (*36*−*39*). Therefore, both the total phenolic content and the ultimate antioxidant activity have been typically underestimated and phytochemicals probably play a larger role than previously thought in total antioxidant activity.

Strawberries contain numerous phenolic compounds, and not all cultivars may contain the same phenolic profile or relative proportions of compounds within the profile; differences in these profiles may subsequently result in complex changes in antioxidant activity or other bioactivities (*11*). In a summary of the phenolic compounds present in different common economic fruit families, it was noted that the flavan-3-ol (+)-gallocatechin was not present in all strawberry cultivars analyzed and that in six out of 14 cultivars tested, (+)-catechin was the sole flavan-3-ol. A single cultivar tested contained equal proportions of (+)-catechin, (−)-epicatechin, and (+)-gallocatechin. Additionally, the relative proportions of the different flavan-3-ols changed unpredictably with the physiological state of the fruit (*11*). Bakker et al. (*40*) found 13 different anthocyanins in a total of 39 strawberry cultivars. Only 14 cultivars contained 10 or more of the anthocyanins identified, and there was variation in relative proportions measured. Fruit phenolics may exhibit antagonistic as well as additive/synergistic activities and interactions with other phenolics and phytochemicals in a particular fruit (*6*, *41*); this balance of synergism and antagonism may be altered dependent upon the relative proportions and existence of particular phenolic compounds. Wang et al. (*19*) measured contents of individual flavonoids isolated from different strawberry varieties and their antioxidant activity, calculating the relative contribution of each compound to the total antioxidant activity as a percentage. Such estimations do not take into account potential interactions between phytochemicals in a mixed system. These interactions are complex and have not been fully characterized and may help to explain our lack of correlations expressed among cultivars. It would be of interest to further explore in more detail the intercultivar differences in specific major and minor phenolic compound content. It is possible that correlations between phenolic content, antioxidative activity, and antiproliferative activity may only be made considering a phenolic concentration range within a particular cultivar or between cultivars containing identical phytochemical profiles.

The phytochemicals present in all strawberry cultivar extracts had a potent inhibitory effect on $HepG_2$ cell proliferation. All eight cultivars exhibited the ability to inhibit cell proliferation in a dose-dependent manner. There was a significant difference among the cultivars with respect to antiproliferative activity, with Earliglow strawberries possessing the highest level of inhibitory action and Annapolis strawberries possessing the lowest level. However, there was no relationship found between antiproliferative activity and total antioxidant activity ($R^2 = 0.463$, $p > 0.05$). Additionally, the total phenolic and flavonoid contents of strawberries did not correlate to antiproliferative activity ($R^2 = 0.332$, $p > 0.05$ and $R^2 = 0.013$, $p > 0.05$, respectively). Similar results were found in a recent study of raspberries by our group (*24*) and may suggest that phytochemicals other than those tested in this experiment are responsible for inhibiting cell proliferation. Alternatively, particular phenolic compounds may act additively and synergistically with some compounds or antagonistically with others and the overall expressed activity may be dependent upon relative proportions of each and/or presence/absence of particular compounds.

Although a linear correlation was not detected between the eight strawberry cultivars tested in this experiment with respect to antioxidant and antiproliferative activities, these measured activities individually differed significantly among cultivars. Because many consumers are now concerned with the health aspects of their food products, this knowledge could be a tool for consumers to estimate the value of each cultivar. Knowledge of the health benefits of antioxidant and antiproliferative activities of different strawberry cultivars could influence which cultivars are commercially sold and used in food products.

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