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Antiproliferative Activity of Apples Is Not Due to Phenolic-Induced Hydrogen Peroxide Formation

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Anticancer compound screening of natural products using tumor cell lines has been commonly used to identify anticancer drugs. Two highly significant anticancer drugs, paclitaxel (Taxol) and camptothecin, were discovered using tumor cell lines by the U.S. National Cancer Institute (NCI) screening program of plants. It has been recently reported that the inhibition of cancer cell proliferation by fruit extracts was indirectly caused by phenolic-induced H_2O_2 production in the cell culture media, suggesting that many previously reported effects of flavonoids and phenolic compounds on cultured cells might be from an artifact of H_2O_2-induced oxidative stress. The objective of the present study was to determine if apple extracts induced H_2O_2 formation in common cell culture media and to investigate if the antiproliferative activity of apple extracts was due to phenolic-induced H_2O_2 formation. It is reported here that apple extracts did not induce H_2O_2 formation in WME, DMEM, or DMEM/Ham F12 media with the cell culture conditions tested. These same extracts inhibited proliferation of $HepG_2$ and Caco-2 cells. Therefore, antiproliferative activity of apple extracts was not due to the phenolic-induced H_2O_2 production in cell culture media. In addition, H_2O_2 added to the culture medium at 100 μM did not cause inhibition of cell proliferation in either $HepG_2$ liver cancer cells or Caco-2 colon cancer cells in vitro.

KEYWORDS: Phytochemical; phenolics; flavonoids; antioxidant activity; cancer

INTRODUCTION

Approximately 60% of all drugs in clinical trials for cancer treatment are either natural compounds or derivatives thereof (*1*). Two highly significant anticancer drugs, paclitaxel (Taxol) and camptothecin, were discovered using tumor cell lines by the U.S. National Cancer Institute (NCI) screening program of plants. Therefore, the importance of screening anticancer compounds from natural products cannot be overestimated. Due to the high costs and time-consuming nature of animal cancer model studies, the initial screening of anticancer compounds from natural products is best accomplished using in vitro cancer cell methods. On the basis of the successes from 1955 to 1984 and the heavy demand for screening, the NCI has developed an anticancer drug-screening program since 1985 using 60 cultured human cancer cell lines to screen chemicals and natural product extracts in vitro to speed the discovery of new anticancer drugs (*1*, *2*).

Regular consumption of fruits and vegetables is associated with a reduced risk for cancer (*3*). Bioactive non-nutrient plant compounds in fruits, vegetables, and grains, known as phytochemicals, have been linked to the reduced risk for major chronic diseases, including cancer and cardiovascular disease (*4*). We have reported that phytochemical extracts from fruits and vegetables have strong antioxidant and antiproliferative activities and proposed that the natural combination of phytochemicals in fruits and vegetables is responsible for the potent antioxidant and antiproliferative activities in those foods (*5*−*7*).

Recently, Lapidot et al. reported that inhibition of cancer cell proliferation by fruit extracts was caused indirectly by phenolic-induced H_2O_2 production in the cell culture medium and concluded that many previously reported effects of flavonoids and phenolic compounds on cultured cells might be from an artifact of H_2O_2-induced oxidative stress (*8*). The objectives of the present study were to (1) determine if apple extracts induce H_2O_2 formation in common cell culture media and (2) investigate if antiproliferative activity of apple extracts was due to phenolic-induced H_2O_2 formation.

MATERIALS AND METHODS

Chemicals and Reagents. Methanol, acetone, and sodium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). Gallic acid was obtained from ICN Biomedical Inc. (Costa Mesa, CA). Folin−Ciocalteu reagent, hydrochloric acid, catalase, phenol red, and horseradish peroxidase were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents used in the study were of analytical grade.

The $HepG_2$ human liver cancer cells and Caco-2 human colon cancer cells were from the American Type Culture Collection (ATCC)

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(Rockville, MD), and the MTS-based cell titer 96 non-radioactive cell proliferation assay was from Promega (Madison, WI). Williams' medium E (WME), Dulbecco's modified Eagle medium (DMEM), Ham's nutrient mixture F-12 medium (Ham F12), and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY). All cell culture media used for H_2O_2 production contained 5% FBS, 10 mM Hepes, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin. WME contained an additional 5 μg/mL insulin, 2 μg/mL glucagon, and 0.05 μg/mL hydrocortisone.

Sample Preparation and Extraction. Red Delicious apples were obtained from Cornell Orchards (Ithaca, NY). The phenolic compounds of apples were extracted using 80% acetone according to the same method reported previously by our laboratory (*5*, *9*). The extract was frozen at −40 °C until analysis. All data collected are reported as means ± SD for at least three replications.

Determination of Total Phenolic Content. The total phenolic content of apple extracts was measured using a modified colorimetric Folin−Ciocalteu method (*9*, *10*). The measurement was compared to a standard curve of gallic acid concentrations. The results were obtained for triplicate extract samples and expressed as milligrams of gallic acid equivalents per 100 g of fresh weight of apples.

Determination of H_2O_2 in Cell Culture Media. The content of H_2O_2 in cell culture medium was determined according to a modification of the method of Pick and Keisari (*11*). Briefly, 0.5 mL of each cell culture medium was thoroughly mixed with 0.5 mL of phenol red/horseradish peroxidase solution (PR/HRPO solution, final concentration = 25 μg of PR/mL and 50 μg of HRPO/mL). The mixture was incubated at 37 °C for 45 min, and the reaction was terminated by adjusting the pH to 12.5 by adding 100 μL of 1 M NaOH. H_2O_2 was measured at 610 nm by absorbance of a stable purple color linearly formed. The recoveries of a known amount of H_2O_2 added to each medium were 105.65 ± 0.97% (n = 5) in WME, 96.2 ± 3.46% (n = 5) in DMEM/Ham F12 (1:1), and 91.44 ± 3.21% (n = 5) in DMEM. The final H_2O_2 concentrations were calculated using a standard curve of H_2O_2 (micromolar) versus absorbance (610 nm) prepared for each medium. Decomposition of H_2O_2 added to each culture medium was measured over a 120 min period at pH 7.4 and 37 °C. The results were expressed as time (minutes) versus H_2O_2 concentration (percent).

Measurement of Inhibition Activity on Cancer Cell Proliferation. $HepG_2$ human liver cancer cells were maintained in WME, containing 10 mM Hepes, 5 μg/mL insulin, 2 μg/mL glucagon, 0.05 μg/mL hydrocortisone, 5% FBS, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin (*12*, *13*). Caco-2 human colon cancer cells were maintained in DMEM, containing 10 mM Hepes, 5% FBS, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin. Both $HepG_2$ and Caco-2 cells were maintained in a 5% CO_2/37 °C incubator.

Antiproliferative activities of apple extracts were measured by the MTS-based cell titer 96 non-radioactive cell proliferation assay described previously (*14*). Cell proliferation was measured by the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfenyl)-2*H*-tetrazolium (MTS) to formazan, which absorbs light at 490 nm. The absorbance was measured at 490 nm using a MRX II DYNEX spectrophotometer (Dynex Technologies, Inc., Chantilly, VA). Briefly, 2.5 × 10^4 $HepG_2$ or Caco-2 cells in growth medium were placed in each well of a 96-well flat-bottom plate. After 4 h of incubation at 37 °C in 5% CO_2, the growth medium was replaced by medium containing apple extracts in concentrations of 0, 5, 10, 20, 30, 40, and 50 mg/mL with or without added catalase (1 Sigma unit/mL). Control cultures received the extraction solution minus the apple extracts, and blank wells contained 100 μL of growth medium alone with no cells. After 96 h of incubation, cell proliferation was determined by the colorimetric MTS assay and results were compared to the control. The effect of added H_2O_2 (0−100 μM) with or without catalase (1 Sigma unit/mL) on the antiproliferative activity was measured as described above for both $HepG_2$ and Caco-2 cancer cell lines. Catalase was also added (1 Sigma unit/mL) to each apple extract treatment to assess antiproliferative activity. At least three replications for each sample were used to determine the inhibitory activity of cell proliferation. The effective median dose (EC_{50}) was determined and expressed as milligrams of apples per milliliter ± SD.

Statistical Analysis. Statistical analysis was conducted using SigmaStat version 8.0 (Jandel Corp., San Rapheal, CA). Differences among treatments were determined using t tests. Means were considered to be significantly different if p values were ≤0.05.

RESULTS AND DISCUSSION

H_2O_2 production was not detected when apple extracts at doses of 0−50 mg/mL (the highest concentration we used in the previous cell culture work) were added to three different cell culture media, WME, DMEM, and DMEM/Ham F12 (1:1), respectively, using the same conditions as reported previously (*5*). The H_2O_2 assay we used is specific for H_2O_2. It is based on the HRPO-mediated oxidation of phenol red by H_2O_2, which results in a linear formation of a stable purple compound at pH 12.5 with an absorbance at 610 nm (*11*). The detection limits of this assay for H_2O_2 were 1 μM in WME with a linear range of 1−80 μM, 5 μM in DMEM/Ham F12 with a linear range of 5−80 μM, and 20 μM in DMEM with a linear range of 40−100 μM (**Figure 1**). This assay has been widely used to measure the H_2O_2 content of cell culture media and tissues (*15*−*17*).

When apple extracts at doses of 0, 5, 10, 20, 30, 40, and 50 mg/mL were added to the WME or DMEM/Ham F12 (1:1) and incubated for 45 min at 37 °C, there was no H_2O_2 production detected at the detection limits of 1 μM for WME and 5 μM for DMEM/Ham F12. Although the detection limit for H_2O_2 in DMEM is 20 μM, no H_2O_2 was detected when compared to the control medium. In addition, there was no indication of dose−response changes when apple extracts at doses of 0, 5, 10, 20, 30, 40, and 50 mg/mL were added to the DMEM and incubated at 37 °C for 45 min. When 47 mg/mL of apple extracts (the value of EC_{50}, a median effective dose, of apple extracts in antiproliferative activity) was added to the WME, DMEM/Ham F12, and DMEM, respectively, there was no H_2O_2 production detected at any time tested after incubation at 37 °C for 0, 15, 30, 45, 60, and 120 min.

Lapidot et al. reported that apple extracts could produce ∼80 μM H_2O_2 (significantly above our minimum detection limits) in their $HepG_2$ medium incubated at 37 °C for 30 min (*8*). The difference between their results and the results reported here might be due to the different methods used to measure the H_2O_2 content. Lapidot et al. used the ferrous oxidation−xylenol orange (FOX2) assay to determine the H_2O_2 content (*8*). The FOX2 assay was originally designed to measure lipid hydroperoxides (ROOHs) (*18*). The principle of the FOX2 is based on the oxidation of ferrous (Fe^{2+}) to ferric ions (Fe^{3+}) by ROOHs (eq 1), and subsequently, Fe^{3+} reacted with xylenol orange to form a complex with a blue-purple color with an absorbance at 560 nm (eq 2).

$$\mathrm{ROOH} + \mathrm{Fe}^{2+} \rightarrow \mathrm{Fe}^{3+} + \mathrm{OH}^- + \mathrm{RO}^{\bullet} \quad (1)$$

$$\mathrm{Fe}^{3+} + \text{xylenol orange} \rightarrow \mathrm{Fe}^{3+}\text{−xylenol orange complex} \quad (2)$$

H_2O_2 is used to calibrate the ROOH content in the FOX2 assay. The FOX2 assay is specific for Fe^{3+} but is not specific for H_2O_2. The increased absorbance is reflective of changes in Fe^{3+} concentrations, not H_2O_2 concentrations. It is reasonable to use H_2O_2 as a reference standard to calibrate the ROOH content in the FOX2 assay (*18*−*21*). However, it is incorrect to interpret the FOX2 assay results as reflecting the H_2O_2 content in cell culture media with numerous components (*8*).

We did not detect any increased absorbance using the FOX2 assay (*18*) when 47 mg/mL of apple extracts (the value of EC_{50} of apple extracts in antiproliferative activity) was added to the

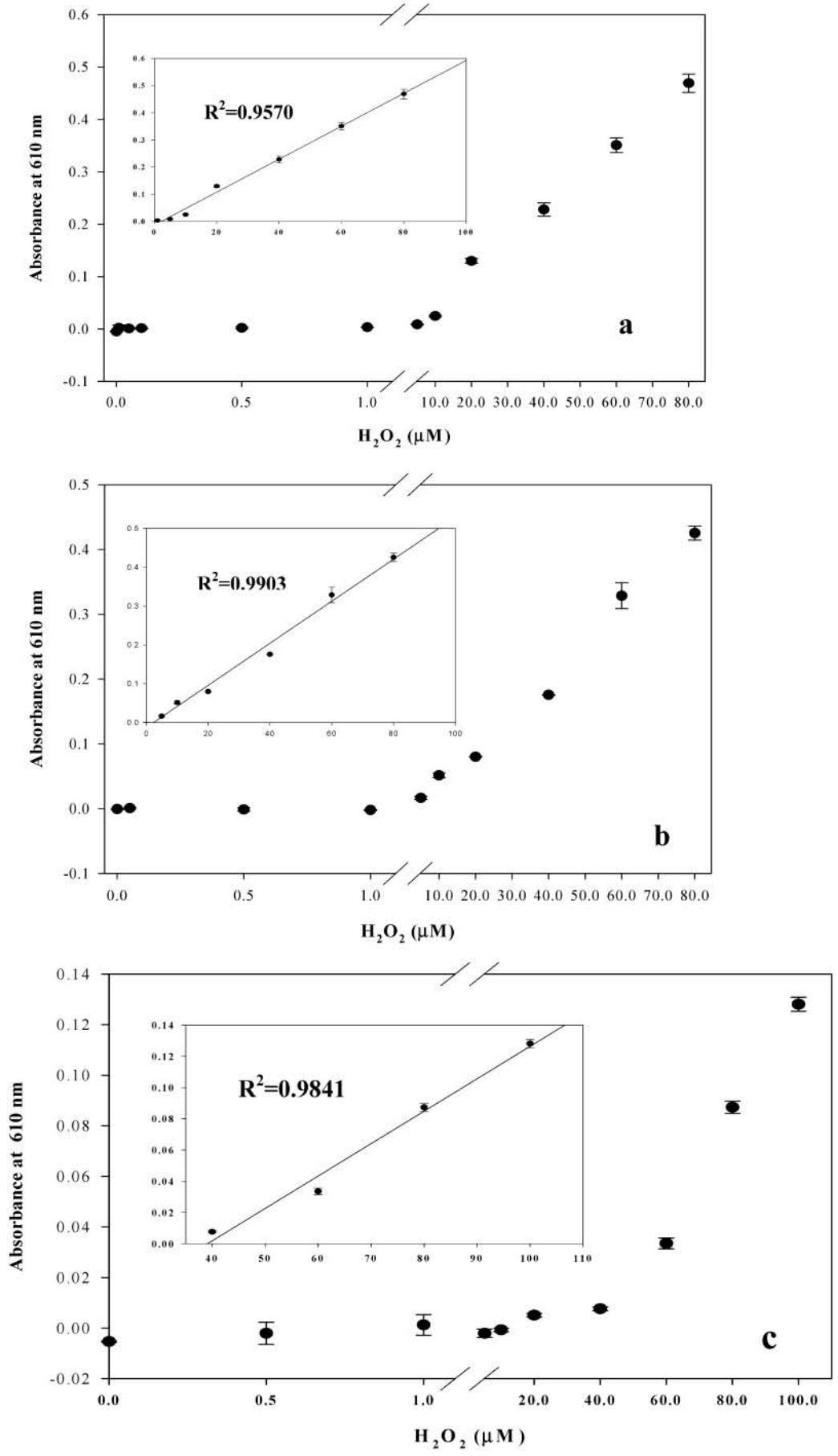

Figure 1. Standard curve for the estimation of H_2O_2 in different cell culture media: (a) WME; (b) DMEM/Ham F12; (c) DMEM (mean ± SD, $n = 3$).

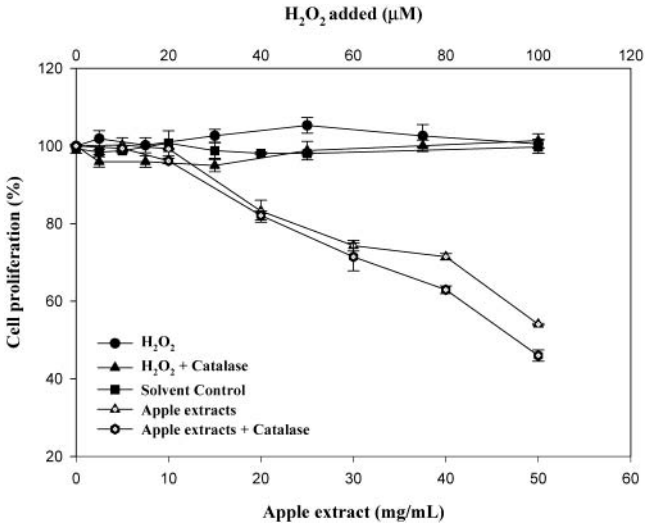

Figure 2. Inhibition of proliferation of $HepG_2$ human liver cancer cells by apple extracts and added H_2O_2 with or without catalase (mean ± SD, $n = 3$).

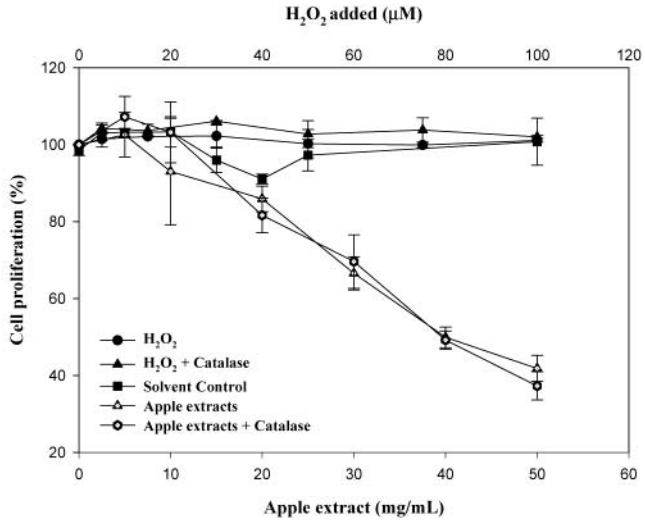

Figure 3. Inhibition of proliferation of Caco-2 human colon cancer cells by apple extracts and added H_2O_2 with or without catalase (mean ± SD, $n = 3$).

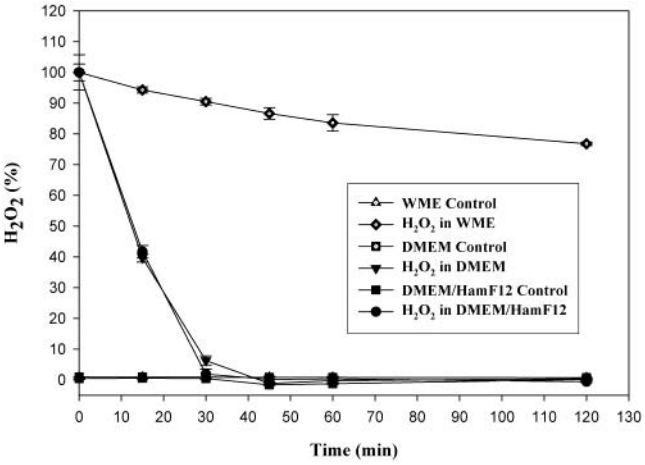

Figure 4. Decomposition kinetics of added H_2O_2 in cell culture media (pH 7.4) at 37 °C (mean ± SD, $n = 3$).

WME, DMEM/Ham F12 (1:1), or DMEM cell cultures, respectively, at any time tested, after incubation at 37 °C for 0, 15, 30, 60, 90, and 120 min. When 50 μM gallic acid was added to the WME, DMEM, or DMEM/Ham F12 (1:1) and incubated at 37 °C for 0, 15, 30, 60, 90, and 120 min, there was no increased absorbance detected in DMEM or DMEM/Ham F12 using the FOX2 assay, but there was a dose−response increase in WME medium. However, we did not detect any H_2O_2 production in gallic acid-added WME medium using the assay based on the HRPO-mediated oxidation of phenol red at the detection limit of 1 μM. Long et al. (*22*) reported that addition of (−)-epigallocatechin, (−)-epigallocatechin gallate, (+)-catechin, and gallic acid to cell culture media resulted in H_2O_2 production using the FOX2 assay, but their media did not contain FBS. Why gallic acid induces an increased absorbance in WME medium using the FOX2 assay is currently under investigation in our laboratory.

Antiproliferative activities of apple extracts and added H_2O_2 with or without catalase on the growth of $HepG_2$ human liver cancer cells in vitro are summarized in **Figure 2**. Apple extracts showed a strong antiproliferative activity toward $HepG_2$ liver cancer cells when compared with the control culture ($p < 0.05$). Cell proliferation was inhibited in a dose-dependent manner after exposure to apple extract concentrations >20 mg/mL with a median effective dose EC_{50} of 56.6 ± 0.29 mg/mL. No cytotoxicity of the apple extracts was seen at any of the concentrations tested. Addition of catalase (1 Sigma unit/mL) did not block the antiproliferative activity when compared to the apple extracts and gave an EC_{50} of 48.7 ± 0.45 mg/mL ($p > 0.05$). These results further indicate that H_2O_2 was not involved in the antiproliferative activity in the cell culture systems tested.

Neither antiproliferative activity nor toxicity was detected when 0−100 μM H_2O_2 was added to the cell culture systems of human liver cancer cells (**Figure 2**). Addition of catalase with H_2O_2 did not change the response of cell proliferation. This further suggested that H_2O_2 was not responsible for the apple extract-induced antiproliferative activity in the cell culture systems. Interestingly, Lapidot et al. did not report if chemically added H_2O_2 inhibited cell proliferation (*8*).

We also tested the effect of apple extracts and added H_2O_2 with or without catalase on the proliferation of Caco-2 human colon cancer cells in vitro (**Figure 3**), and results similar to those obtained with $HepG_2$ cells were observed. Apple extract showed antiproliferative activities on Caco-2 human cancer cell growth in a dose-dependent manner starting at concentrations >10 mg/mL with a median effective dose (EC_{50}) of 42.5 ± 2.64 mg/mL. Addition of catalase (1 Sigma unit/mL) did not block the antiproliferative activity when compared to the non-catalase-treated apple extracts with an EC_{50} value of 39.2 ± 1.13 ($p > 0.05$). Neither was antiproliferative activity detected when 0−100 μM H_2O_2 was added to the cell culture medium for Caco-2 colon cancer cells (**Figure 3**). Addition of catalase with H_2O_2 did not change the response of cell proliferation or have any significant differences when compared to H_2O_2-treated samples and the control ($p > 0.05$).

The decomposition kinetics of H_2O_2 in WME, DMEM, and DMEM/Ham F12 (1:1) are shown in **Figure 4**. When 80 μM H_2O_2 was added to the WME and incubated for 120 min at 37 °C, a slight decomposition was observed. However, when 80 μM H_2O_2 was added to the DMEM and DMEM/Ham F12 and incubated at 37 °C, a more rapid decomposition curve was observed in both media. The time for a 50% H_2O_2 loss ($t_{1/2}$) was 14.0 min for DMEM and 12.9 min for DMEM/Ham F12 (**Figure 4**). The cause of the decomposition of H_2O_2 in both DMEM and DMEM/Ham F12 is not clear, but it is worth further

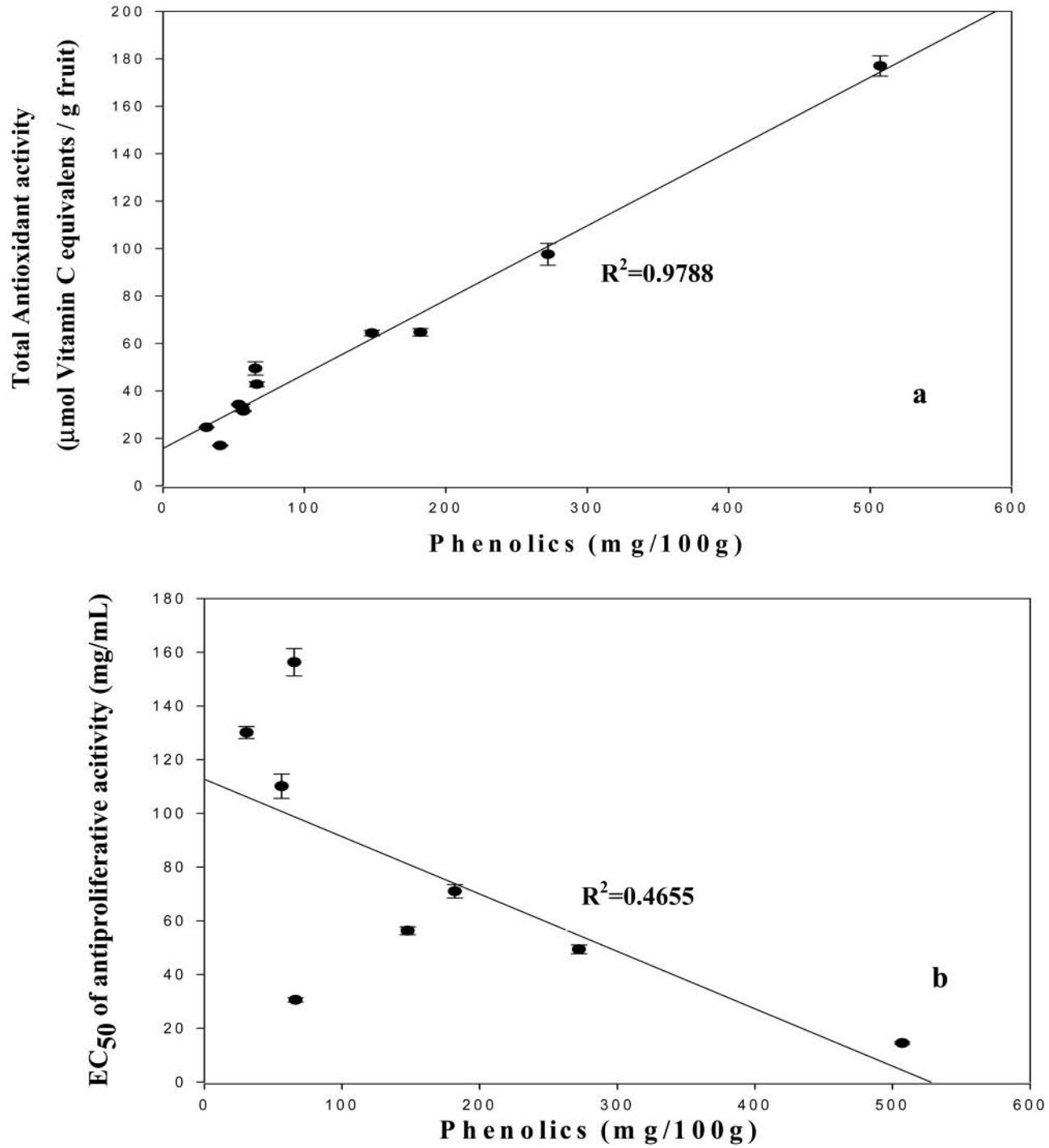

Figure 5. Relationship between total antioxidant activity (A), antiproliferative activity (B), and total phenolic content in selected common fruits (mean ± SD, $n = 3$).

investigation. Although Caco-2 cells were not exposed to the full amounts of H_2O_2 added due to the quick loss of H_2O_2 in the media, $HepG_2$ liver cancer cells were exposed to H_2O_2 for periods long enough to potentially affect the system if H_2O_2 was reactive in the system. Therefore, it is reasonable to conclude that H_2O_2 at the level of 100 μM did not affect the proliferation of $HepG_2$ liver cancer cells in the cell culture systems as shown in **Figure 2**.

Antioxidant activity and the inhibition of tumor cell proliferation are two different concepts. The phytochemicals in apples other than ascorbic acid seem to significantly enhance their antioxidant properties and their capacity to inhibit the proliferation of tumor cells in vitro (*5*). There is a direct relationship between phenolic content and antioxidant activity in common fruits ($r^2 = 0.9788$, $p < 0.05$) (**Figure 5a**) (*7*). However, there is no relationship between phenolic content and antiproliferative activity ($r^2 = 0.4655$, $p > 0.05$) (**Figure 5b**). If antiproliferative activity resulted from phenolic-induced H_2O_2 production, we would expect a correlation between phenolic content and antiproliferative activity. In addition, the inhibition of cancer cell proliferation by fruit extracts cannot be explained by the total phenolic contents of the fruits tested (*7*). This suggests that a specific phenolic compound or a class of phenolics in fruits are responsible for their antiproliferative activities. Therefore, further identification of specific phytochemicals for their antiproliferative activities is needed.

The antiproliferative activities of some fruits and vegetables are significantly different in $HepG_2$ liver cancer cells and Caco-2 colon cancer cells. For example, plum extract has a strong activity against $HepG_2$ liver cancer cells but a low activity against Caco-2 colon cancer cells (*23*). This suggests that the putative anticancer compound or compounds in plums require hepatic activation to have anticancer activity. If antiproliferative activity resulted from phenolic-induced H_2O_2 production as Lapidot et al. suggested (*8*), we should expect the plum extract to have similar activity against both liver cancer cells and colon cancer cells. The plum results also suggest that different bioactive compounds are present in various foods and that they may act at different locations. We are currently following up on these preliminary results.

Raspberries were shown to have potent inhibitory activity against the proliferation of $HepG_2$ human liver cancer cells (*14*). Kiwigold and Goldie raspberry varieties are genetically identical to the Heritage raspberry variety except for a single-point mutation that affects anthocyanin synthesis. The Heritage raspberry has a higher phenolic content and a higher antioxidant activity than the lighter colored Kiwigold and Goldie varieties. Antiproliferative activities were the same for all three varieties. Pigment content is a factor affecting antioxidant activity and phenolic content but is not a factor in the inhibition of cell proliferation. The raspberry results also do not support the results of Lapidot et al. If antiproliferative activity resulted from phenolic-induced H_2O_2 production as Lapidot et al. suggested, we would expect the three raspberry varieties to have different antiproliferative activities. Therefore, we hypothesize that in raspberries other phytochemicals, not anthocyanins, are responsible for the inhibition of tumor cell proliferation. We have ongoing experiments to identify the individual chemical compounds that inhibit proliferation.

In summary, cell proliferation assays have been widely and successfully used to screen anticancer compounds in conjunction with cytotoxicity assays (*1*, *2*, *5*, *6*) and will continue to serve as an important model for initial anticancer drug screening from natural products. The key is to separate the effective dose (EC_{50}) from the cytotoxicity dose. Apple extracts did not induce H_2O_2 formation in WME, DMEM, and DMEM/Ham F12 with the cell culture conditions tested. Antiproliferative activity of apple extracts was not due to the phenolic-induced H_2O_2 production in cell culture media as Lapidot et al. reported (*8*). In addition, H_2O_2 at 100 μM did not cause inhibition of cell proliferation in $HepG_2$ liver cancer cells in vitro. These data confirm our original conclusion (*5*) that apple extracts exhibit strong antiproliferative activity. This activity could be due to the inherent combination and complex interaction of phytochemicals in apples, not due to phenolic-induced H_2O_2 production as indicated by Lapidot et al. (*8*).

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