

# Inhibition of Growth and Induction of Differentiation of Colon Cancer Cells by Peach and Plum Phenolic Compounds

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**Abstract.** *The action of extracts from anthocyanin-enriched plums and peaches on growth and differentiation was studied with human colon cancer cells. Growth inhibitory effects were observed in Caco-2, SW1116, HT29 and NCM460 cells. In Caco-2 cells but not in the other cells studied there was evidence for increased differentiation as judged by increased activity of alkaline phosphatase and dipeptidyl peptidase. A differentiating effect on Caco-2 cells was not seen with cyanidin or cyanidin-3-glucoside but the action of the fruit extracts was additive with the action of butyrate and with the MEK1/2 inhibitor U0126. Fractionation using C18 indicated activity resided within a fraction containing anthocyanins but further fractionation using LH-20 suggested that most of the activity was in a fraction containing polyphenols other than anthocyanins. It was concluded that several peach and plum phenolic molecules can influence growth and differentiation in human colon cancer cells.*

There is evidence in the literature that plant extracts containing polyphenolic molecules including anthocyanins can have growth inhibitory effects on cancer cells (1-8). Molecules of this type can possess antioxidant (9, 10) and cancer chemopreventive action (11-15). In the present work, we examined extracts from plum and peach varieties that have a high anthocyanin content (16). Our objective was to study

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both growth inhibition and the potential to exert a differentiating effect on colon cancer cells in culture. Differentiation was judged by an increase in enzyme markers. Having established such effects, we sought to establish if they could be attributed to anthocyanins or might reflect the action of other polyphenolic molecules. To obtain an indication of the specificity of the changes for transformed colon cells, we compared the actions of different phenolic fractions on Caco-2, SW1116 and HT29 cells with effects on the minimally transformed human colon cell line, NCM460 (17).

## Materials and Methods

*Cells and determination of cell proliferation.* SW1116, HT29 and Caco-2 human colon cancer cells were incubated at 37°C in RPMI-1640 medium with 5% fetal calf serum and 25 mM *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) buffer. NCM460 human colon cells were maintained in M3:10 medium from INCELL Corp., San Antonio, TX, USA. The incorporation of [<sup>3</sup>H]thymidine into DNA was measured after incubating cells for 2 hours with 2 microcuries [<sup>3</sup>H]thymidine as described elsewhere (18).

*Fruit extracts and fractionation.* Methanolic extracts were prepared from fruit of the following genotypes: Flame Prince and BY00P6653 peach and Black Splendor plum as described by Vizzoto *et al.* (16). In addition, methanolic extracts were similarly derived from the Black Amber plum variety. Subsequent fractionation of the methanolic extracts on C18 was performed using the PrepSep C18 system from Fisher Scientific, Pittsburgh, PA, USA with sequential elution with ethyl acetate (fraction 1) and methanol containing 5% formic acid (fraction 2). Further resolution of the fractions eluted from C18 was carried out by sequential elution from Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) with ethanol, methanol and finally with 70% acetone.

*Assay of phenolic molecules and antioxidant activity.* The procedure for the assay of total anthocyanin content was adapted from the procedure of Fuleki and Francis (19) as described in (20). Total

Table I. Total anthocyanins, total phenolics and antioxidant activity in peach and plum varieties (derived from reference 16).

| Fruit               | mg/100 g Fresh tissue |           |                      |
|---------------------|-----------------------|-----------|----------------------|
|                     | Anthocyanins          | Phenolics | Antioxidant activity |
| Flame Prince peach  | 3±1                   | 158±14    | 113±15               |
| BY00P6653 peach     | 118±8                 | 523±28    | 660±20               |
| Black Splendor plum | 227±69                | 372±14    | 242±16               |

Table II. Inhibition of [<sup>3</sup>H]thymidine incorporation into DNA in Caco-2 and NCM460 cells by fruit extracts and anthocyanins.

| Incubation                 | Thymidine incorporation (% Control) |         |
|----------------------------|-------------------------------------|---------|
|                            | Caco-2                              | NCM460  |
| Control                    | 100                                 | 100     |
| Black Splendor (2.5 mg/ml) | 46±13**                             | 13±2**  |
| Flame Prince (2.5 mg/ml)   | 64±4                                | 64±7*   |
| BY00P6653 (2.5 mg/ml)      | 26±7**                              | 1±1**   |
| 50 µM Cyanidin 3-glucoside | 58±27*                              | 69±23   |
| 50 µM Cyanidin             | 50±17*                              | 53±10** |

Cells were incubated for 24 hours in the presence or absence of fruit extracts or anthocyanins before addition of [<sup>3</sup>H]thymidine and incubation for a further 2 hours. The data are given as the mean±S.D for 4 determinations. \*p<0.05, \*\*p<0.01.

phenolics were determined using the Folin-Ciocalteu reagent using chlorogenic acid as a standard (20). Antioxidant activity was quantified by the 2,2-diphenyl-1-picrylhydrazyl radical method using Trolox as a standard as described (20).

**Enzyme assays.** A total of 1.5×10<sup>6</sup> cells were incubated with 10 ml RPMI-1640 medium with 25 mM HEPES and 5% fetal calf serum. The medium was changed after 24 hours and unless stated otherwise the cells were incubated for 72 hours before harvesting. The cells were washed with phosphate-buffered saline and extracted with 0.5% NP40, 0.25 M NaCl, 5 mM EDTA and 50 mM Tris (pH 8.0). The protein concentration of the extract was determined using the BCA Protein Assay Reagent from Pierce, Rockford, IL, USA. Enzymes were assayed at 37°C as described elsewhere (21). Alkaline phosphatase was assayed in a volume of 0.22 ml using *para*-nitrophenyl phosphate (4.5 mM) as substrate and 6.8 mM 2-amino-2-methyl-1-propanol (pH 10.3) as the buffer. Incubations were for 30 minutes and were stopped by the addition of 2.0 ml 0.05 N NaOH. Formation of product was monitored by the change in absorbance at 410 nm. Aminopeptidase and dipeptidyl peptidase were assayed in a volume of 0.5 ml using 2 mM L-alanyl-4-nitroanilide or 2 mM Gly-Pro-4-nitroanilide as substrates, respectively. The buffer was 0.1 M Tris (pH 8.0). Incubations were stopped by the addition of 2.0 ml 1 M acetate (pH 4.2). Production of the product, 4-nitroaniline, was monitored at 405 nm.

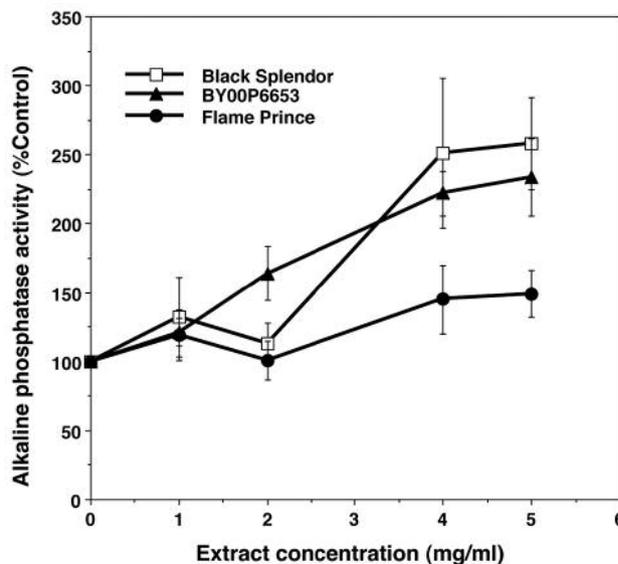


Figure 1. Effect of incubation with fruit extracts for 72 hours on the specific activity of alkaline phosphatase in Caco-2 cells.

Table III. Percentage of unacetylated histone H4 in Caco-2 cells after incubation with butyrate or fruit extracts for 24 hours.

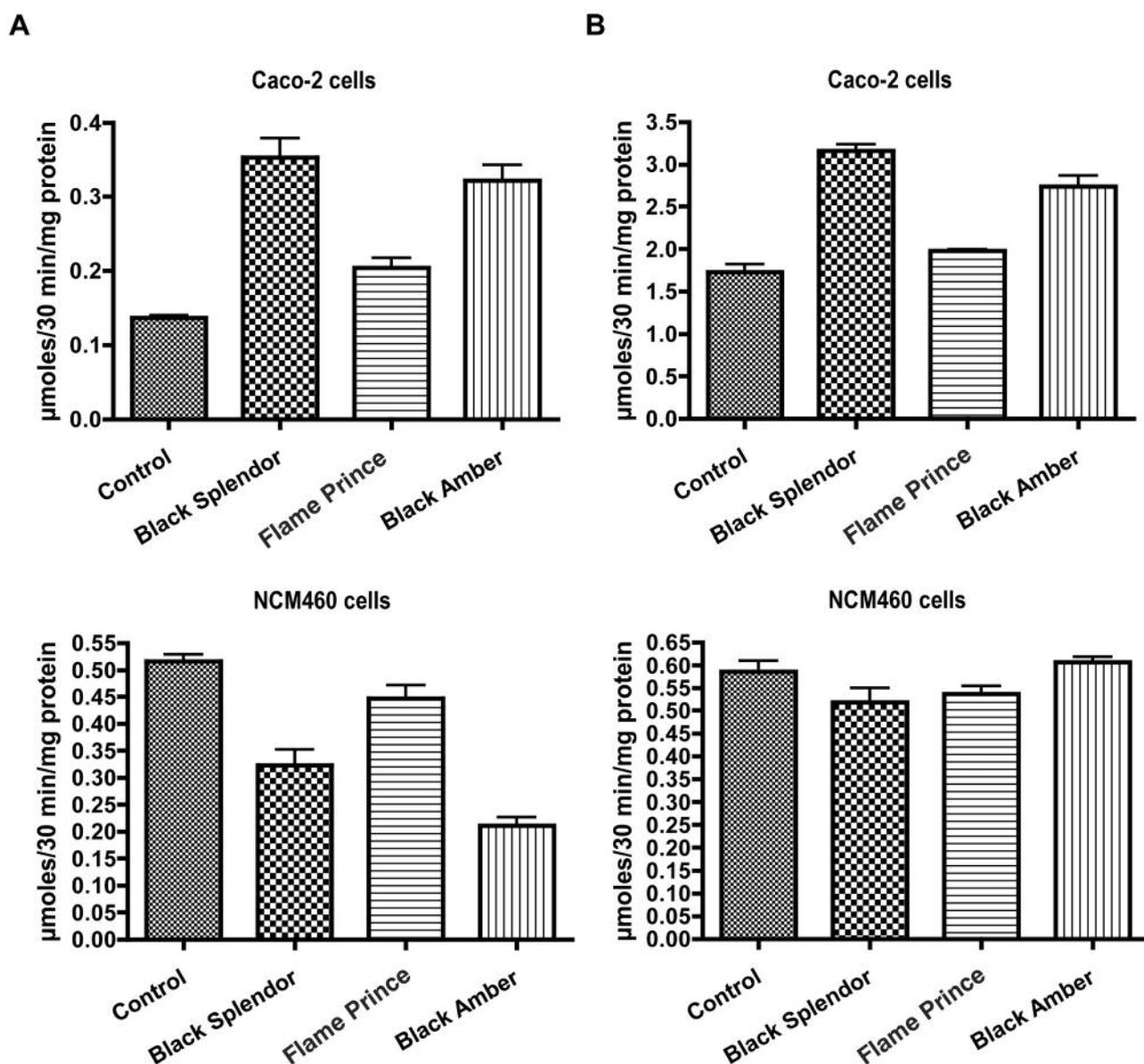
| Treatment                 | Unacetylated H4 histone as a percentage of total H4 histone |
|---------------------------|---|
| Control                   | 46%   |
| 0.5 mM Butyrate           | 7%  |
| Black Amber (10 mg/ml)    | 48%   |
| Black Splendor (10 mg/ml) | 44%   |
| BY00P6653 (10 mg/ml)      | 47%   |

**Histone acetylation.** The isolation of histones and electrophoresis on urea-acetic acid polyacrylamide gels was performed as described elsewhere (22). The relative levels of acetylated H4 histones were quantified by densitometry of Coomassie-blue-stained gels.

**Statistical evaluation.** Statistical significance of the results was determined by a two-tailed Student's *t*-test or by Dunnett's test for multiple comparisons using the Instat program (GraphPad Software, San Diego, CA, USA). A probability of less than 5% was considered significant.

## Results

The anthocyanin and phenolic contents and the antioxidant activity for two peach and one plum variety are presented in Table I. It may be noted that anthocyanins constitute a different proportion of the total phenolics for the three fruits and that the antioxidant activity correlates more closely with the total phenolic content than with the anthocyanins.

Figure 2. *continued*

In our initial studies, Black Splendor plum and BY00P6653 peach methanolic extracts at 1 mg per ml inhibited [ $^3\text{H}$ ]thymidine incorporation into DNA of Caco-2 and NCM460 cells but significant effects were not seen with the low-anthocyanin Flame Prince peach extract at that concentration. The data in Table II indicate that at 2.5 mg/ml, the extract from BY00P6653 peach caused greater inhibition of [ $^3\text{H}$ ]thymidine incorporation in Caco-2 and NCM460 cells than did the extracts from Black Splendor plum or Flame Prince peach and that the degree of inhibition was similar in the two cell lines. Cyanidin and cyanidin-3-glucoside at 50  $\mu\text{M}$  were less inhibitory for [ $^3\text{H}$ ]thymidine incorporation

than the fruit extracts, although the extracts had lower anthocyanin concentrations.

Black Splendor and BY00P6653 extracts at concentrations greater than 1 mg/ml caused an increase of alkaline phosphatase activity in Caco-2 colon cancer cells but not in NCM460, HT29 or SW1116 colon cancer cells. However, protein synthesis was reduced in all four cell types. The dose dependence of the increase in alkaline phosphatase activity in Caco-2 cells after incubation with Black Splendor plum and BY00P6653 peach extracts is illustrated in Figure 1. There were minimal effects when the Caco-2 cells were incubated with the Flame Prince peach extract.

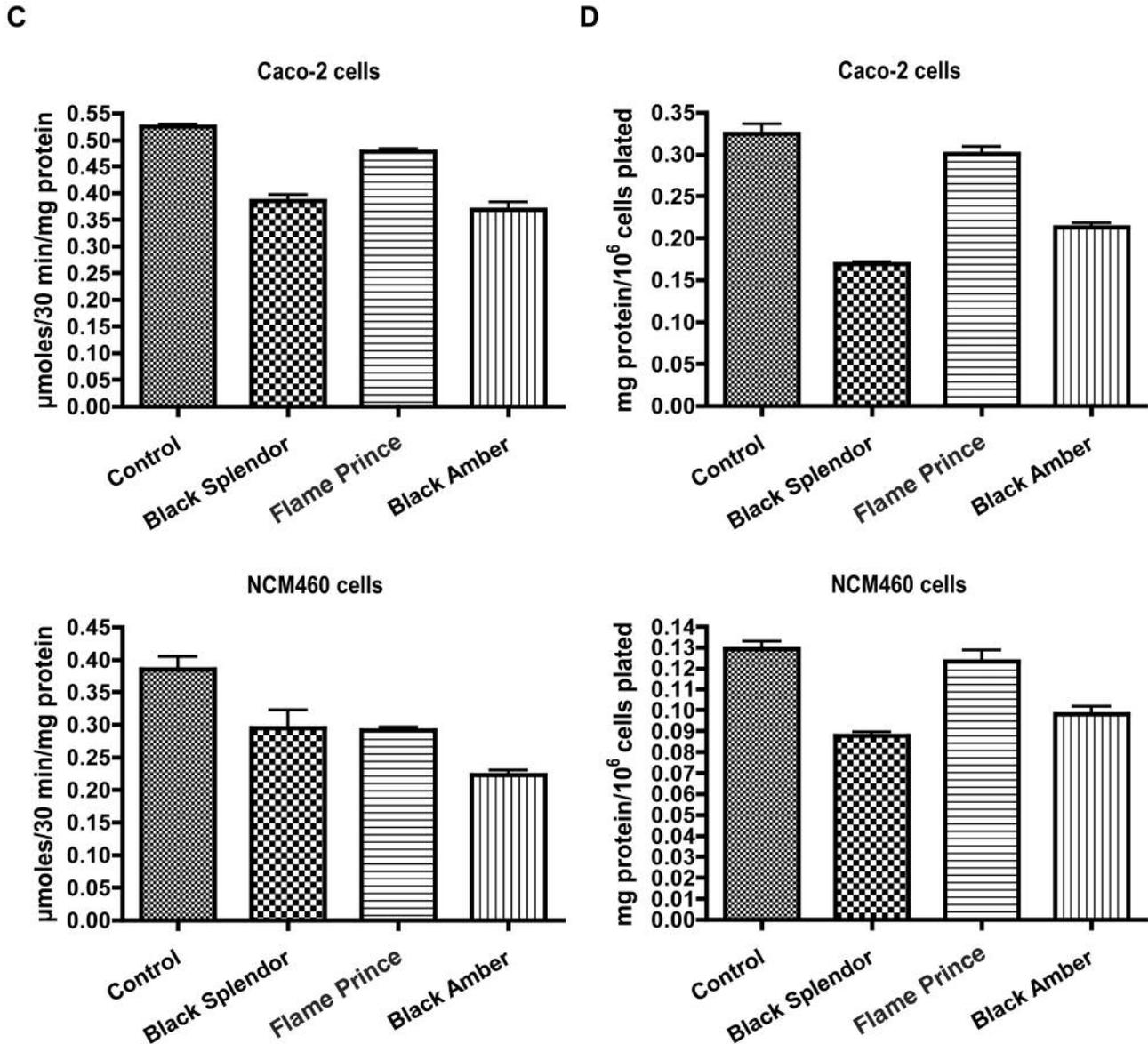


Figure 2. Comparison of the effects of incubation of Caco-2 and NCM460 cells with fruit extracts (5 mg per ml) for 72 hours on the specific activity of A, alkaline phosphatase; B, dipeptidyl peptidase; C, aminopeptidase; D, protein yield.

The results in Figure 2A indicate that the increased alkaline phosphatase activity seen in Caco-2 cells contrasted with a decrease in activity in NCM460 cells when they were incubated with the Black Splendor and Black Amber plum extracts. The Black Splendor plum was derived from the Black Amber variety and shows a higher anthocyanin content (4.3 µg per mg extract *versus* 1.7 µg per mg extract for Black Amber). Comparison of Black Amber and Black Splendor plum extracts revealed that both could induce alkaline phosphatase and to a lesser extent dipeptidyl peptidase activity (Figure 2B) in Caco-2 cells. Increased

activities of dipeptidyl peptidase were seen when Caco-2 cells were incubated with the fruit extracts but there was no increase in aminopeptidase activity (Figure 2C). The amount of protein extracted from the cells after the 72-hour incubation suggested growth inhibitory effects with the Black Splendor and Black Amber plum extracts in both Caco-2 and NCM460 cells (Figure 2D).

When Caco-2 cells or NCM460 cells were incubated with 20 or 40 µM cyanidin, or 20 µM cyanidin-3-glucoside, there were little or no effects on alkaline phosphatase activities or total protein levels (data not shown).

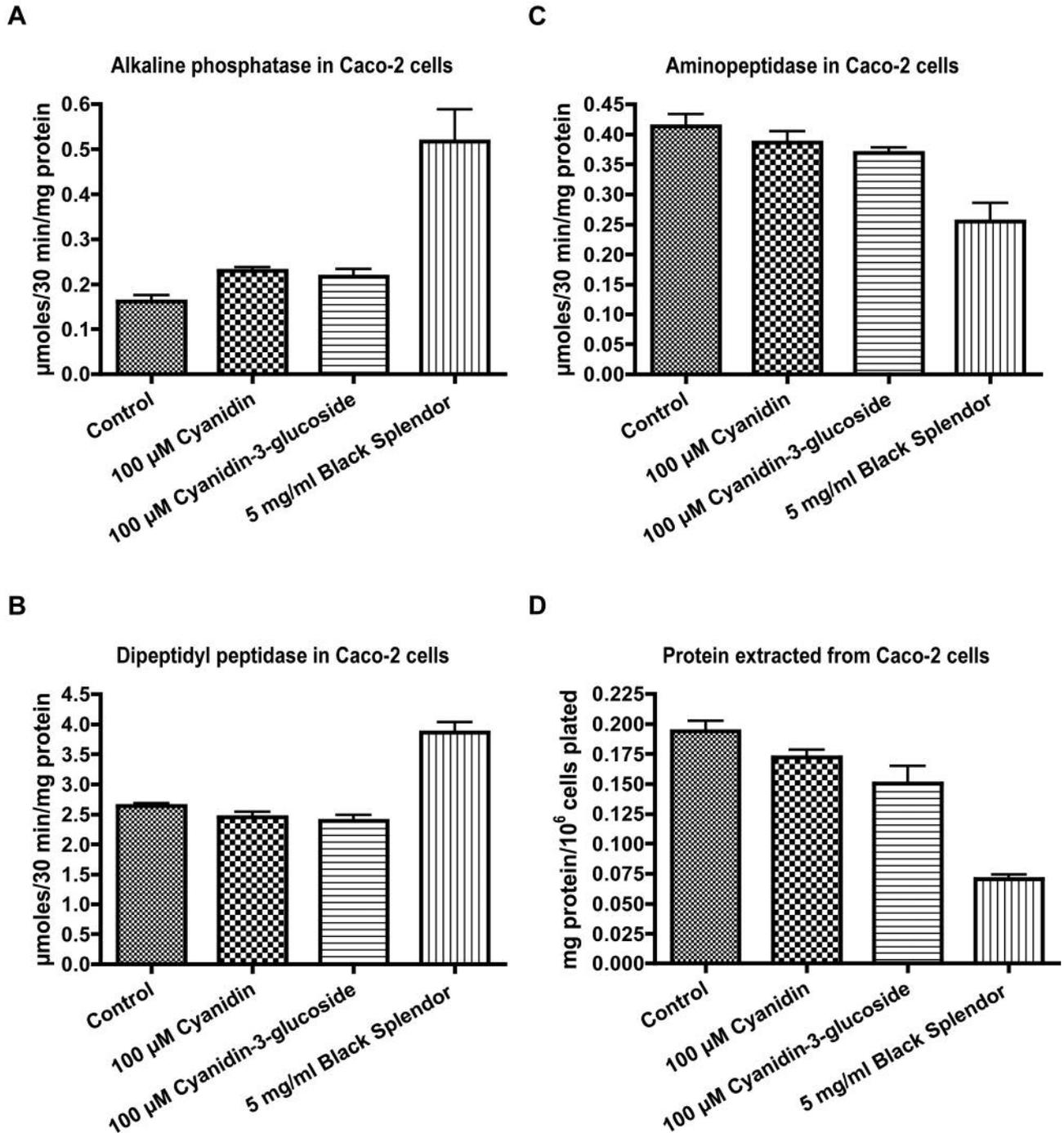


Figure 3. Effect of incubation of Caco-2 cells for 72 hours with cyanidin, cyanidin-3-glucoside and Black Splendor plum extract on enzyme activities and protein yield.

Increasing the concentration of cyanidin and cyanidin-3-glucoside to 100 µM had only a limited effect on induction of alkaline phosphatase in Caco-2 cells and for all the parameters examined there were greater effects with the Black Splendor extract (Figure 3 A-D).

Additivity or synergism was seen for the increase in alkaline phosphatase activity when Caco-2 cells were incubated with 0.5 mM butyrate and Black Splendor plum extract (2.5 mg/ml) either as single agents or in combination (data not shown). Similarly, an increase was seen with 0.5

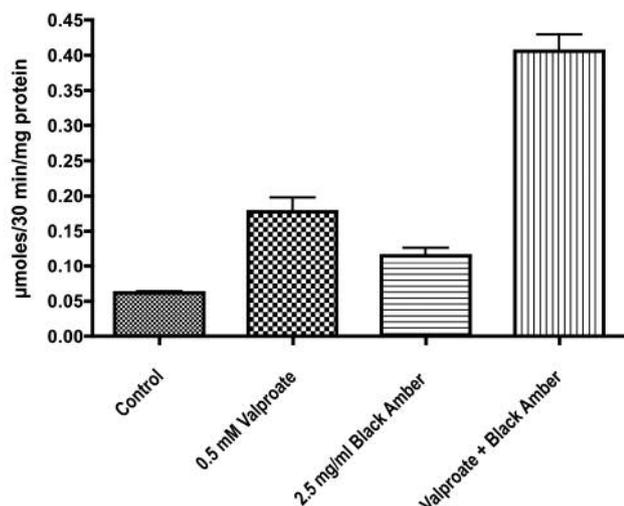


Figure 4. Additive effect of incubation of Caco-2 cells with valproate and Black Amber plum extract on alkaline phosphatase activity.

mM valproate and the Black Amber plum extract (2.5 mg/ml) either as single agents or in combination (Figure 4). Thymidine incorporation in Caco-2 cells was inhibited by Black Amber and Black Splendor extracts at 2.5 and 5.0 mg/ml to a similar extent with the two extracts and to a greater degree than was seen with NCM460 cells (Table II). Thymidine incorporation in HT29 cells was inhibited more by the BY00P6653 peach extract than by the Black Amber or Black Splendor plum extracts (data not shown).

The induction of alkaline phosphatase by incubation with 0.5 mM butyrate was enhanced by the fruit extracts in Caco-2 cells but not in SW1116, HT29 and NCM460 cells (data not shown). In contrast to the induction of H4 histone acetylation seen after incubation of Caco-2 cells with 0.5 mM butyrate, there was no effect on histone acetylation after incubation with the plum and peach extracts (Table III).

An additive effect on the alkaline phosphatase activity in Caco-2 cells was seen after incubation with the MEK1/2 inhibitor U0126 and either the Black Amber or Black Splendor extract but the induction of alkaline phosphatase in NCM460 cells by U0126 was not enhanced by the plum extracts (data not shown). A study with the Black Amber plum extract is illustrated in Figure 5.

The plum and peach extracts were eluted from a C18 column with ethyl acetate (fraction 1) and methanol containing 5% formic acid (fraction 2). Anthocyanins were eluted in the second fraction and it was fraction 2 that caused induction of alkaline phosphatase activity when incubated with Caco-2 cells. Additivity in the effect on alkaline phosphatase activity was seen when Caco-2 cells were co-incubated with Black Splendor fraction 2 and 0.5 mM butyrate (Figure 6) but this was not apparent with the other

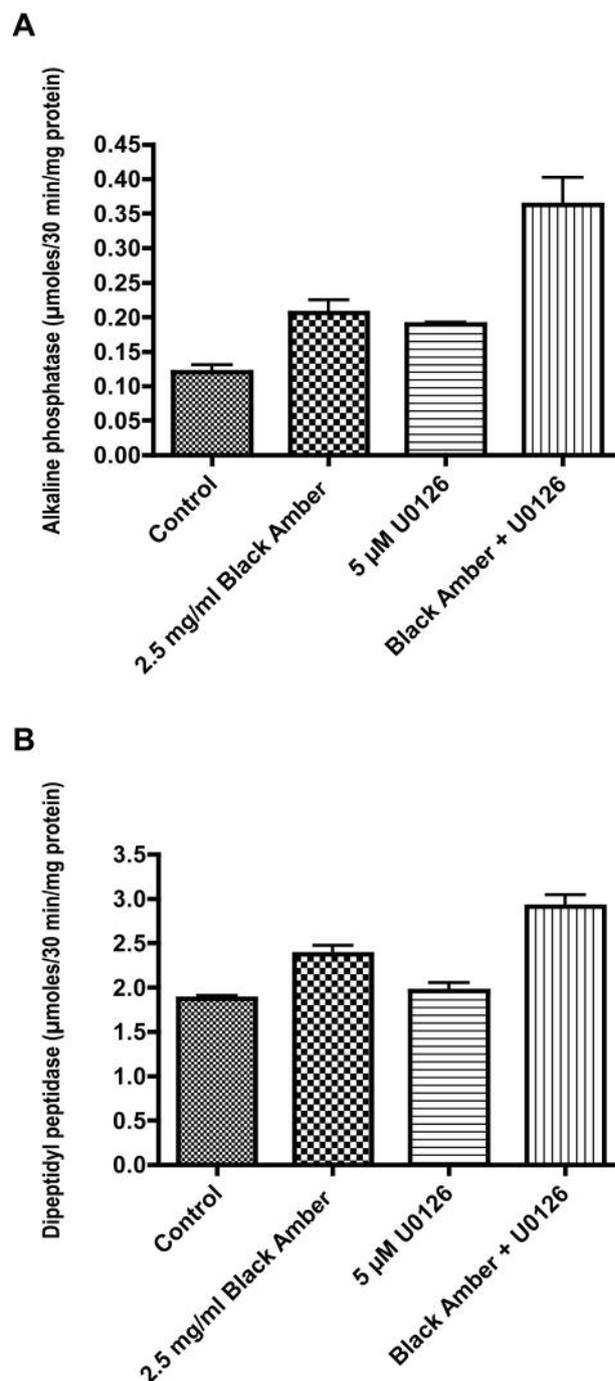


Figure 5. Additive effects of incubation of Caco-2 cells with Black Amber plum extract and U0126 for 72 hours on the activities of A, alkaline phosphatase and B, dipeptidyl peptidase.

cell lines examined (data not shown). A combination study with Caco-2 cells incubated with butyrate and BY00P6653 fraction 2 is presented in Figure 7. The data indicate an additive increase in alkaline phosphatase activity, with a less pronounced effect on dipeptidyl peptidase activity.

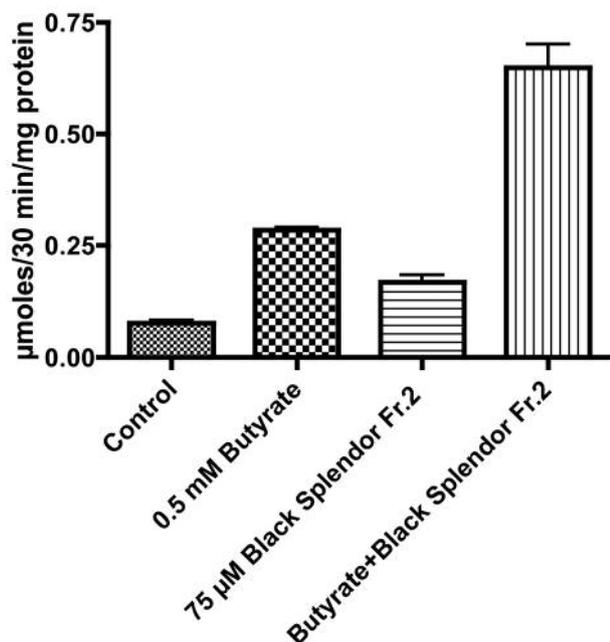


Figure 6. Additive effect of incubation of Caco-2 cells with butyrate and fraction 2 of Black Splendor plum extract (75 µM anthocyanin concentration) on alkaline phosphatase activity.

Fraction 2 eluted from a C18 column was further fractionated on an LH-20 column with sequential elution with ethanol, methanol and 70% acetone. Solvent was evaporated and the solutes were redissolved in the incubation medium. Most of the anthocyanins were eluted with ethanol but it was the eluate with 70% acetone that was generally most inhibitory for thymidine incorporation into DNA and for decreasing protein synthesis when incubated with the different colon cancer cell lines. The 70% acetone eluate was the most effective fraction for increasing the activities of alkaline phosphatase and dipeptidyl peptidase when incubated with Caco-2 cells (Figure 8A, B). Aminopeptidase activity was not significantly affected under these conditions (Figure 8C) but the amount of protein decreased after incubation with the fractions eluted with methanol and 70% acetone (Figure 8D). Increased enzyme activities were not seen with the other cell lines (data not shown) but, as illustrated for HT29 and SW1116 cells in Figure 9, there was an inhibition of [<sup>3</sup>H]thymidine incorporation when incubated with the 3 eluates with the most potent being the 70% acetone eluate.

## Discussion

Anthocyanins by virtue of their red and blue colors are among the more obvious plant phenolic molecules. They have been studied for their antioxidant properties and for their potential anticancer effects. Fruits such as plums and peaches differ in their content of anthocyanins and other phenolic

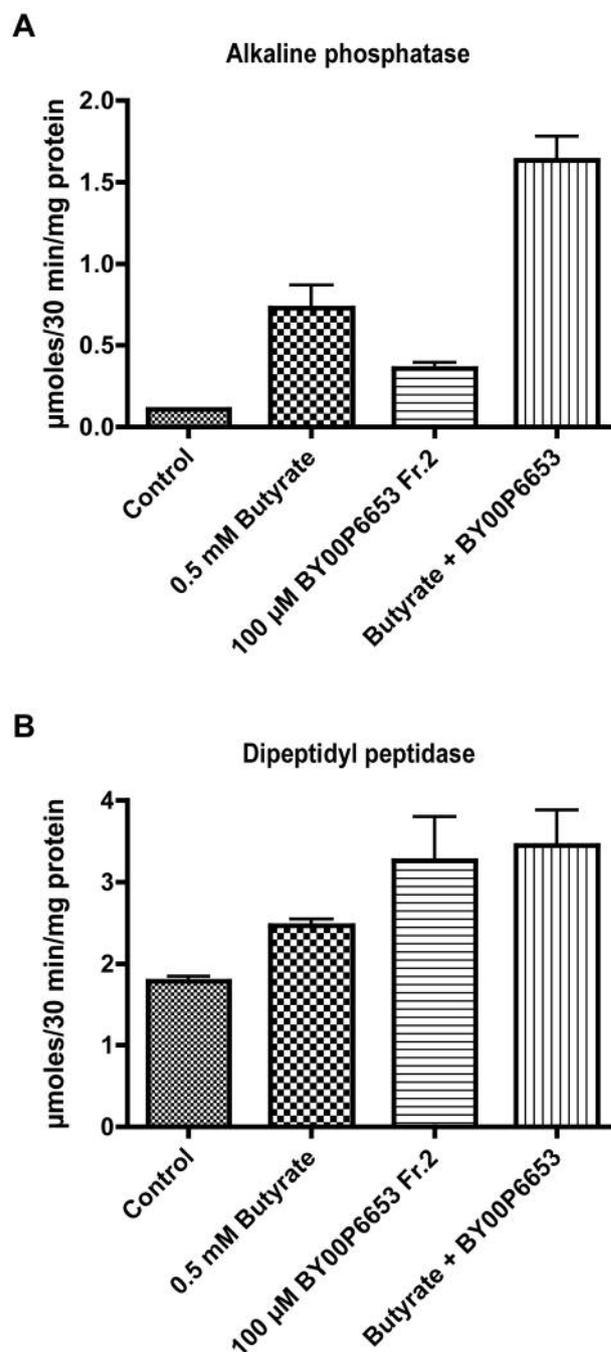


Figure 7. Combined effect of butyrate and BY00P6653 peach extract fraction 2 when incubated for 72 hours with Caco-2 cells on A, alkaline phosphatase and B, dipeptidyl peptidase. The concentration of the BY00P6653 peach extract fraction 2 is expressed in terms of anthocyanin concentration.

molecules. Selective cultivation of fruit-bearing plants that are enriched for polyphenolic molecules presents the prospect of obtaining varieties with enhanced chemopreventive potential against cancer.

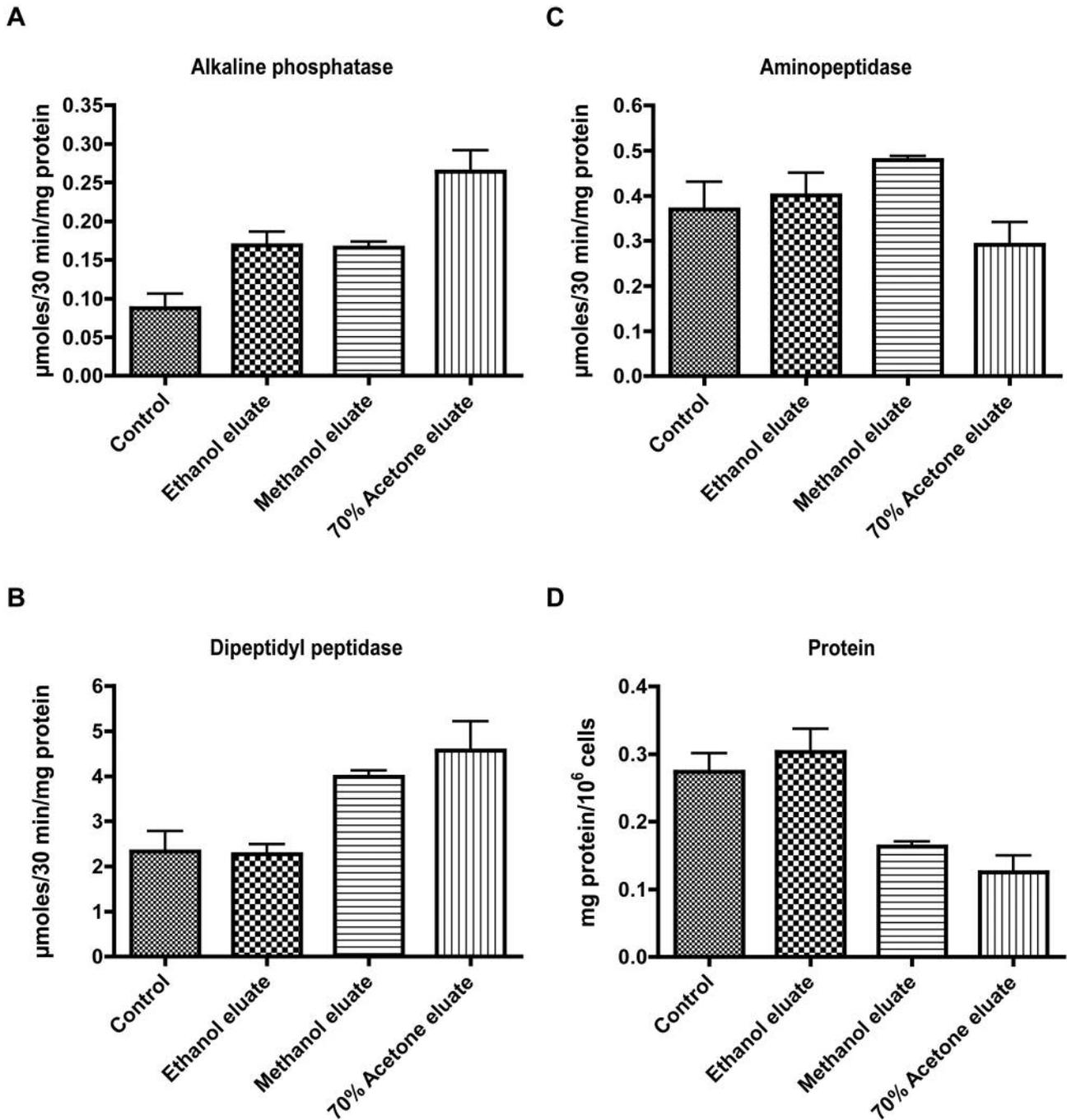


Figure 8. Effects of subfractions from BY00P6653 peach extract fraction 2 on enzyme activities and protein yield in Caco-2 cells after incubation for 72 hours. The eluates from fraction 2 were obtained from an LH-20 column and were incubated with cells at a polyphenol concentration of 50 µg chlorogenic acid equivalents per ml.

Alkaline phosphatase activity has been used as a marker for differentiation in colon cancer cells, particularly using the Caco-2 cell line. One of the most potent inducers of alkaline phosphatase activity in these cells is butyrate, an inhibitor of histone deacetylase. The action of butyrate can be increased

by inhibitors of the mitogen-activated protein (MAP) kinase pathway such as U0126 (21, 23, 24). The increase in alkaline phosphatase activity after incubation with the fruit extracts studied was different from the effect of butyrate in that it was not accompanied by an increase in histone acetylation and it

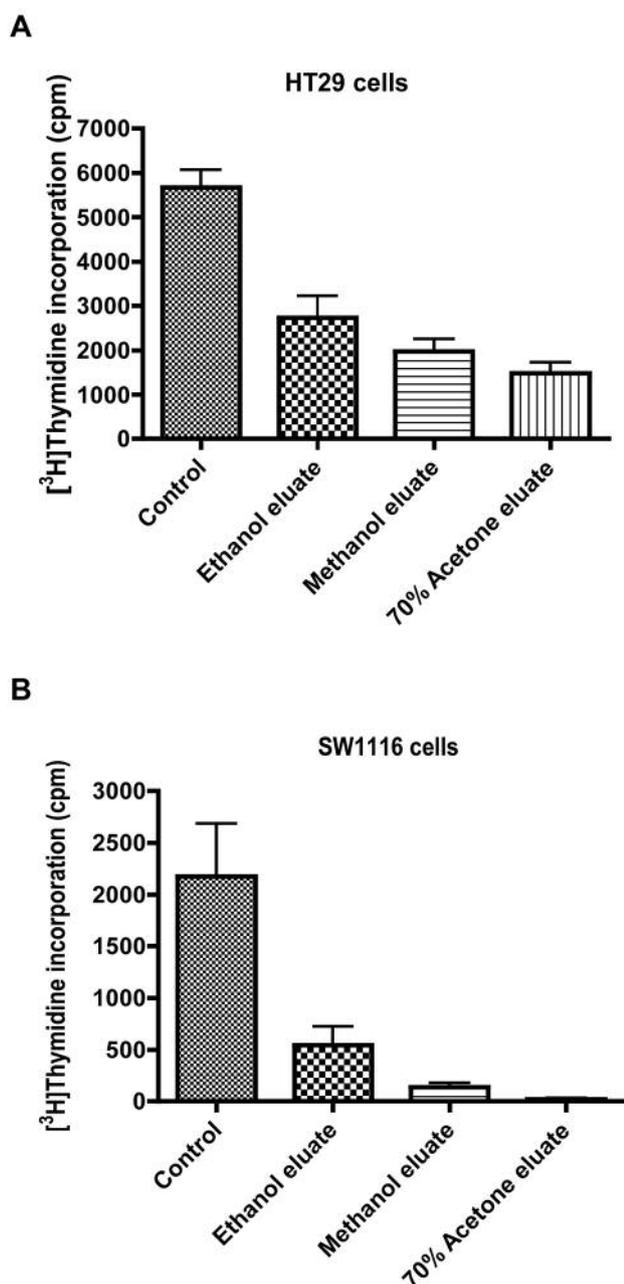


Figure 9. Inhibition of [<sup>3</sup>H]thymidine incorporation into DNA of A, HT29 and B, SW1116 cells after incubation of subfractions of BY00P6653 peach extract fraction 2 eluted from an LH-20 column. Incubation with polyphenol fractions were conducted at a concentration of 100 µg chlorogenic acid equivalent per ml for 72 hours.

did not occur in all the cell lines that were studied. The action of the fruit extracts may more closely resemble that of a MAP kinase pathway inhibitor such as U0126 and effects of anthocyanin containing fruit extracts on the MAP kinase pathway in other systems have been reported (25). Synergistic interactions between MEK/ERK pathway

inhibitors and histone deacetylase inhibitors have been reported in different tumor types (26, 27). In contrast to the increases that were seen in alkaline phosphatase and dipeptidyl peptidase, there was little change in aminopeptidase activity when cells were treated with the polyphenolic extracts.

The initial report on the NCM460 colon cell line suggested that it had untransformed properties (17). NCM460 cells have been reported to be more resistant to anthocyanin-induced cell cycle perturbations than transformed colon cancer cells (28, 29). However, in our studies, inhibition of growth in NCM460 cells by anthocyanin-containing extracts was similar to that seen in colon cancer cells. We have previously reported data indicating that the properties of NCM460 cells, including levels of alkaline phosphatase and dipeptidyl peptidase activity, change with continued passage of these cells in culture (21). However, growth inhibitory effects were observed with the plum and peach extracts even in early passages of the NCM460 cells.

Although the fruit extracts examined were taken from fruits cultivated for increased anthocyanin content and early studies indicated association of activity with fractions containing anthocyanins, further fractionation indicated that polyphenolic molecules other than anthocyanins may be more significant for the induction of differentiation-associated enzyme activities. Individual anthocyanins (cyanidin and cyanidin-3-glucoside) did not exert differentiating effects under conditions in which they inhibited cell proliferation. Of the 3 colon cancer cell lines examined, the most responsive was the Caco-2 cell line. It remains for subsequent investigation to determine which individual polyphenolic molecules can exert the most potent differentiating and growth inhibitory activity in colon cancer cells.

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