JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Inhibitory Effect and Mechanisms of an Anthocyanins- and Anthocyanidins-Rich Extract from Purple-Shoot Tea on Colorectal Carcinoma Cell Proliferation

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ABSTRACT: One newly bred variety of tea cultivar, purple-shoot tea, was selected to evaluate its antiproliferative effects on colorectal carcinoma cells, as well as normal colon cells. The phytochemicals and identified catechins of purple-shoot tea extract (PTE) were significantly higher than that of ordinary tea, especially the anthocyanins (surpassed by 135-fold) and anthocyanidins (surpassed by 3.5-fold). PTE inhibited the proliferation of COLO 320DM (IC_{50} = 64.9 μg/mL) and HT-29 (IC_{50} = 55.2 μg/mL) by blocking cell cycle progression during the G_0/G_1 phase and inducing apoptotic death. Western blotting indicated that PTE induced cell cycle arrest by reducing the expression of cyclin E and cyclin D1 in COLO 320DM and the upregulation of p21 and p27 cyclin-dependent kinase inhibitors in HT-29. Two cells treated with PTE also indicated the cleavage of PARP, activation of caspase 3, and an increased Bax/Bcl-2 ratio. Our results showed that PTE is a potential novel dietary agent for colorectal cancer chemoprevention.

KEYWORDS: *apoptosis, cell cycle, colorectal carcinoma, purple-shoot tea*

INTRODUCTION

Colorectal cancer represents a major public health problem that has progressively increased to be the third most common cancer diagnosed in both men and women in Taiwan.[1] The incidence of colorectal cancer is highly affected by diet, suggesting that colorectal cancer is a suitable target disease for dietary agents. Natural products have become more popular for the prevention or treatment of cancer. Teas with high levels of catechins have been consumed as a favorite beverage worldwide and are regarded as health drinks with cancer preventative properties.[2,3] Tea catechins can scavenge reactive oxygen species and thereby directly or indirectly exhibit a broad spectrum of biological, pharmacological activity against the proliferation of carcinoma via modulating important cellular signaling processes.[4−7] The end result of these effects may be the inhibition of tumor cell growth, induction of apoptosis, or the inhibition of angiogenesis.[3] Studies have shown that tea catechins and proanthocyanidins can induce G_0/G_1 phase cell cycle arrest in several human tumor cell lines with no detectable toxic effects.[6,8] However, some of the tea flavonoid levels did not directly correlate with anticarcinogenic activities.[9] Anthocyanins are the water-soluble pigments found in many plant species including grapes and berries, contributing their attractive red, orange, blue, and purple colors.[10,11] Interest in anthocyanins has recently increased owing to their potential health benefits and pharmacological activity, which include antioxidant and antiproliferative properties.[12,13]

Specialty tea varieties with purple- or red-colored buds and leaves have been shown to contain high quantities of anthocyanidins and anthocyanins.[14,15] Although it was suspected that the newly selected cultivars of newly bred teas in Taiwan, such as the purple-shoot tea clone, might be rich in anthocyanidins and anthocyanins, their characteristics and anticolorectal activity had not been studied. The presence of anthocyanidins and anthocyanins in addition to the catechins have been expected to contribute to even more effective activity.[16] The purpose of this study was to examine the inhibitory effect of PTE on the proliferation of colorectal cell lines. Two human colorectal carcinoma (CRC) cell lines (COLO 320DM and HT-29) and a rat small intestine epithelial cell line (IEC-6) were treated with PTE and assessed for viability, cell cycle distribution, apoptosis, and changes in the protein levels involved in cell cycle control. In addition, we compared the effectiveness of purple-shoot tea versus ordinary tea.

MATERIALS AND METHODS

Materials. Folin-Ciocalteu reagent and standards such as gallic acid (98%), caffeic acid, myricetin, quercetin, catechin (98%), (−)-epicatechin (EC) (98%), (−)-epigallocatechin (EGC) (98%), (−)-epicatechin gallate (ECG) (98%), (−)-gallocatechin gallate (GCG)

Received: November 11, 2011
Revised: February 9, 2012
Accepted: March 10, 2012
Published: March 11, 2012

(98%), (−)-epigallocatechin gallate (EGCG) (95%), delphinidin, cyanidin, malvidin, and peonidin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) media 1640, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), L-glutamine, trypsin, and antibiotics were purchased from Gibco Ltd. (Paisley, UK). Proteinase inhibitor cocktail, sodium orthovanadate, sodium fluoride (NaF), sodium pyrophosphate, Triton X-100, ammonia persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), Tween 20, and Rhodamine 123 were procured from Sigma (St. Louis, MO, USA). The bicinchoninic acid (BCA) protein assay reagent was from Pierce (Rockford, IL, USA). The acrylamide was from Bio-Rad (Hercules, CA, USA). The polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was from Millipore (Bedford, MA, USA). The mouse monoclonal anticaspase 3, Bcl-2, cyclin A, cyclin D1, and cyclin E antibodies were from Zymed (San Francisco, CA, USA). The goat polyclonal antipoly [ADP-ribose] polymerase (PARP), and Bax antibodies, as well as the goat antirabbit, antimouse, and rabbit antigoat secondary antibodies conjugated with horseradish peroxidase (HRP) were from R&D Systems (Minneapolis, MN, USA). The X-ray film was from Fuji (Tokyo, Japan). The annexin V conjugated with fluorescein isothiocyanate (FITC) was from Gene Research (Taipei, Taiwan). All other chemicals were of analytical purity grade.

Cell Lines. The human CRC cell lines HT-29 and COLO 320DM, and the rat small intestine epithelial cell IEC-6 were obtained from the Bioresource Collection and Research Center, Taiwan. The HT-29 cells, derived from the tumor of a patient with grade II colon adenocarcinoma, were cultured in 90% RPMI medium 1640 supplemented with 10% heat-inactivated FBS. The COLO 320DM cells, derived from a moderately differentiated adenocarcinoma of a colon cancer patient, were cultured in the same medium as HT-29. The IEC-6 was cultured in 90% DMEM supplemented with 0.1 Unit/mL bovine insulin and 10% FBS. All media used here were supplemented with 25 U/mL penicillin and 25 μg/mL streptomycin as antibiotics. The cells were incubated at 37 °C in a 95% air/5% CO_2 and water-saturated atmosphere. All experiments were carried out on cell lines passaged 5−20 times.

Preparation of Sample. The purple-shoot tea and TTES No. 12 tea cyanine came from the Tea Research and Extension Station (Taiwan). TTES No. 12 tea from green-colored tea clones, a popular tea ordinarily used for processing Oolong tea in Taiwan, served as the control in this study. Young tender shoots, comprised of the youngest two leaves plus a bud, were harvested. The fresh tea leaves were freeze-dried until a constant weight was achieved. All of the tea was milled into a powder using a grinder (RT-02, Rong Tsong Iron Factory, Taipei, Taiwan), and the powder was sealed and stored at −20 °C or processed to extract the polyphenol compounds. The powder was then extracted twice with a 50-fold volume of methanol for 60 min while being vigorously shaken. After the samples were filtered with Whatman No. 1 filter papers, the filtrates were evaporated under a vacuum below 50 °C using a rotary evaporator (CCA-1111, EYELA, Japan) to remove the methanol, and then freeze-dried (Yamato, Freeze-Dryer DC 400, Tokyo, Japan). The methanolic extracts of the purple-shoot tea and TTES No. 12 tea were defined as PTE and TTE.

Measurement of Phytochemicals. The amount of total phenolics and condensed tannin in the PTE was determined by colorimetric assay as reported previously.[17] The total flavonoids contents were measured according to Zhishen et al.[18] Both the flavonoids and the condensed tannin are expressed as milligrams of catechin equivalents per gram of dry weight. Anthocyanins were quantified by the pH differential method,[19] with cyanidin-3-glucoside used as the reference standard. Anthocyanidin content was determined by spectrophotometry at 550 nm, on the basis of a colorimetric reaction with 10% $NH_4Fe\ (SO_4)_2$.[20] Anthocyanidins content was expressed in terms of milligrams of cyanidin chloride equivalents per gram of extract solid.

HPLC Analysis of the Catechins and Anthocyanidins. The catechins and anthocyanidins were characterized by HPLC using a Shimadzu SCL-LC 10A HPLC fitted with a SIL 10AD autosampler (Shimadzu, Kyoto, Japan). Chromatography was performed with an ODS HYPERSIL (Thermo Fisher Scientific, Suwanee, GA) reverse phase column (25 cm × 0.46 cm i.d., 5 μL) and a UV−vis detector (Shimazu, Kyoto, Japan). The mobile phase of the catechins assay contained 1% acetic acid (solvent A) and acetonitrile (solvent B), with a linear gradient from A/B (92:8) to A/B (73:27) over a period of 40 min with a flow rate of 1 mL/min. The detector was set at 280 nm. The mobile phase of the anthocyanidins assay contained 10% formic acid (solvent A) and acetonitrile (solvent B), with a linear gradient from A/B (92:8) to A/B (70:30) over a period of 50 min with a flow rate of 1 mL/min. The detector was monitored at 520 nm.

Cell Proliferation Assay. Two CRC cell lines (HT-29 and COLO 320DM) and the rat small intestine epithelial cell line (IEC-6) were plated at 100,000 cells in 60 mm tissue culture dishes. After 18 h of culture, the cells were treated with different concentrations of DMSO-dissolved PTE (0, 25, 50, 100, 200, or 400 μg/mL). At 24 and 48 h, the cells were collected by trypsinization, stained with trypan blue, and the cell number in suspension was counted in duplicate using a hemocytometer. Data were the average of three independent experiments.

Cell Cycle Analysis. The cell cycle distribution of PTE-treated cells was measured by the DNA contents in each cell using flow cytometry.[21] The percentages of G_1, S, and G_2/M cell cycle phases were determined using Modfit software (Verity Software House, Inc., Topsham, ME, USA).[22]

Apoptosis Analysis. Apoptosis measurement was carried out using annexin V to label the cell surface phosphatidylserine of the apoptotic cells.[21,22] Briefly, the treated cells were trypsinized and stained with annexin V conjugated with FITC. Flow cytometry measured the fluorescence intensity with the FL-1H channel detecting FITC. The untreated cells served as the negative control.

Immunoblotting. The alteration of the protein profile involved in the cell cycle and apoptosis in the CRC cells was assessed by immunoblotting.[21,23] Briefly, the cells were washed with ice-cold phosphate-buffered saline and lysed in a homogenization buffer (10 mM Tris-HCl at pH 7.4, 2 mM EDTA, 1 mM EGTA, 50 mM NaCl, 1% Triton X-100, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1:100 proteinase inhibitor cocktail) on ice for 30 min. After centrifugation for 30 min at 13,000 rpm at 4 °C to remove insoluble materials, the protein concentration of the lysate was determined using a BCA protein assay kit and then separated on SDS-PAGE. The resolved bands were electrotransferred to PVDF membranes using a semidry blot apparatus (Bio-Rad). Immunoblotting was performed by incubating the PVDF membranes with 5% nonfat milk in Tris-buffered saline supplemented with Tween 20 (TBST, 10 mM Tris, pH 7.4, 150 mM NaCl, and 0.2% Tween 20) for 1 h at room temperature to block the residue-free protein binding sites on the PVDF. The membrane was incubated with different primary antibodies in 3% nonfat milk in TBST at 4 °C for 18 h. After repeating the washing with TBST, the membrane was incubated with secondary antibodies conjugated with HRP. The immunoblot were developed using enhanced chemiluminescence, and the luminescence was then visualized on X-ray film.

Statistical Analysis. All data are expressed as means ± standard deviation (SD) unless otherwise stated. Differences between the groups were calculated using the Student's unpaired *t*-test. The dose-dependent effect was calculated using simple linear regression. $P < 0.05$ was considered statistically significant. All statistical analysis was performed using SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Phytochemical Content. To study the antiproliferation effects of the flavonoids present in the PTE on the colon carcinoma cell line, a proximate analysis of the phytochemicals was done. The total phenolic content, total flavonoids, condensed tannin, anthocyanins, and anthocyanidins of the PTE are shown in Table 1. Both tea extracts contained high concentrations of phenolic compounds and were rich in flavonoids, condensed tannins, anthocyanins, and anthocyani-

Table 1. Phytochemical Content of PTE and TTE[a]

phytochemicals	concentration (mg/g)	
	PTE	TTE
total phenol	200.3 ± 13.3	157.9 ± 15.7
total flavonoids	61.7 ± 2.5	48.2 ± 3.9
condensed tannin	160.4 ± 17.9	62.2 ± 1.9
anthocyanins	3.73 ± 0.02	0.03 ± 0.00
anthocyanidins	77.6 ± 4.0	22.1 ± 4.0
caffeic acid	67.2 ± 0.3	50.5 ± 2.1
gallic acid	0.7 ± 0.1	0.6 ± 0.0
EC	48.9 ± 0.1	10.1 ± 1.9
ECG	72.32 ± 1.3	24.3 ± 0.5
EGC	163.1 ± 0.5	75.7 ± 1.5
EGCG	319.8 ± 0.4	243.3 ± 1.2
quercetin	nd	nd
myricetin	6.1 ± 0.1	3.7 ± 0.2

[a]All data are expressed as the mean ± standard deviation of at least three experiments; nd means not detected.

dins relative to other edible leaves. However, the PTE appeared to contain significantly more anthocyanidins and anthocyanins than the TTE, results similar to those of the Xiao et al. study on purple bud tea in China in 2009.[24] They determined that the total contents of anthocyanidins, tea polyphenols, water extracts, catechins, and complex catechins were higher in varieties of purple-buds as compared to the common tea varieties in China. When we compared their findings to ours, we noted that, as predicted, there were abundant anthocyanins in the PTE, 135 times more than in the TTE. Anthocyanins biosynthesis has been demonstrated to be upregulated when the plant is exposed to irradiation.[25] The reduction in the risk of photoinhibition on purple-shoot tea provides advantages that may explain the natural occurrence of anthocyanidins- and anthocyanins-rich teas at the high altitudes or in the hot climates of Taiwan. Purple leaves of purple-shoot tea containing high quantities of anthocyanins can be exposed to higher ambient radiation than the green leaves of ordinary tea. Therefore, purple-shoot tea could grow well when exposed to the strict radiation from sunlight in Taiwan's summer.

The identifications of catechins and flavonoids were determined by HPLC, according to the retention times obtained from authentic standards run under identical conditions. The major catechins in both tea leaves include: (+)-catechin (C), gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG), with EGCG being the most abundant in the tea (Table 1). All of the contents of the various catechins and flavonoids in the PTE were higher than in the TTE. Anthocyanidins are common plant pigments. Four anthocyanidins compounds have been tentatively identified by HPLC. The PTE contained delphinidin, cyanidin, malvidin, and peonidin. No known anthocyanidins compounds of the most major peak have been identified in the chromatogram to date in the present study, although the most predominant anthocyanidin in the Kenyan tea was malvidin.[14]

Effect of PTE on Cell Proliferation. Despite the numerous health-enhancing properties of teas, no work had been done to investigate the anticolorectal activity of these colored polyphenols in newly bred purple-shoot tea clones. First investigated was the effect of PTE on the colorectal cell survival ratio. As compared to the untreated cells, the cell survival ratio decreased in a dose-dependent manner in two colorectal cancer cell lines after 24 h (Figure 1). The sensitivity of these two

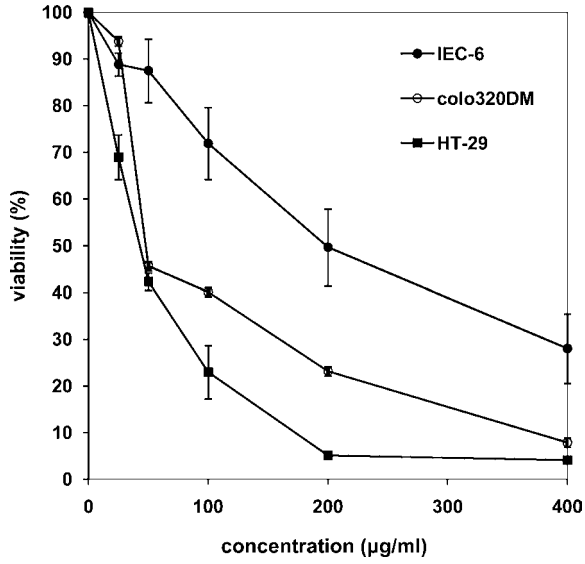

Figure 1. Dose-dependent response of the HT29 cells, COLO 320DM cells, and IEC-6 cells to PTE. A total of 10^5 cells in 60 mm dishes were treated with increasing concentrations of PTE as indicated, and then incubated at 37 °C for 24 h. The viable cells were trypsinized, stained with trypan blue, and counted under a microscope. Cell viability was expressed as a percentage of untreated cells. Data are the average of three independent experiments and expressed as means ± SD.

carcinoma cell lines to the PTE in this assay was similar, but the HT-29 cells exhibited greater sensitivity to the PTE than the COLO 320DM cells ($P < 0.01$). The low cytotoxicity of the PTE treated on the normal intestinal epithelium IEC-6 cells when the concentration was below 100 μg/mL concentration. Malik et al. discovered that the anthocyanins-rich extract from chokeberry specifically inhibited the growth and cell cycle progression, but little to no effect was observed on the growth of normal colon cells. The anthocyanins- and anthocyanidins-rich purple-shoot tea showed similar results in the present study.[26] The colonies of HT-29 were inhibited to lower than 50% of the control level by PTE treatments of more than 55.2 ± 10.8 μg/mL. The IC_{50} of the PTE on HT-29 was significantly lower than that of the TTE with 262.5 ± 13.5 μg/mL. The colonies of COLO 320DM of PTE were inhibited to lower than 50% of the control level by PTE treatments of more than 64.9 ± 7.1 μg/mL, lower than that of the TTE at 132.3 ± 31.0 μg/mL. These results demonstrated that PTE exhibited a significant inhibitory effect on the proliferation of these two CRC cell lines. The inhibitory efficacy of purple-shoot tea was higher than ordinary green tea in Taiwan.

Cell Cycle Arresting Properties of PTE. To determine the cellular mechanism of growth inhibition of PTE on the CRC cells, we investigated cell cycle progression after the PTE treatment. With the cell cycle phases at 100 μg/mL, the G_1 phase increased, and the S phase decreased in the HT-29 and COLO 320DM (Figure 2A,B). The treated cells showed a blockage at the G_1 phases of the cell cycle. Various studies have shown that tea extracts, which are catechins or theaflavins, inhibited the proliferation of colorectal cancer cells due to their ability to stop the G_1,[27,28] S,[27] or G_2/M[29] phase arrest of the cell cycle. In this study, we showed that PTE at a concentration

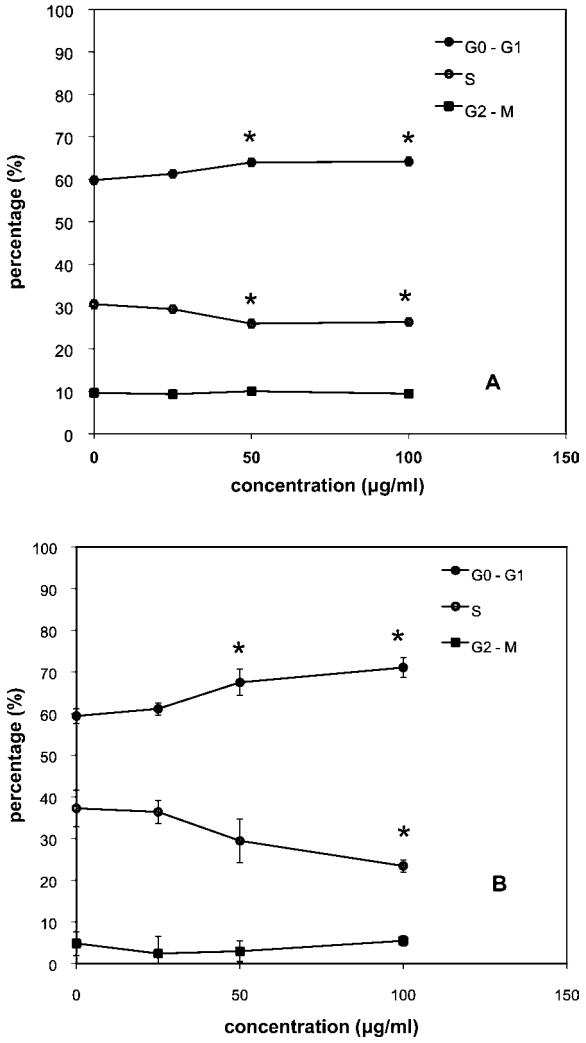

Figure 2. Cell cycle analysis of PTE-treated cells. HT-29 cells (A) and COLO 320DM (B) cells were treated with increasing concentrations of PTE as indicated and then incubated at 37 °C for 24 h. Cells were harvested and fixed in 70% alcohol and then stained with propidium iodide as described in Materials and Methods. The stained cells were analyzed using a flow cytometer, and the distribution in each cell cycle phase was determined by Modfit software. Data are expressed as a percentage of total cells, represent the averages of three independent experiments, and are expressed as mean ± SD. The asterisk (*) represents a significant difference as compared to 0 μg/mL ($P < 0.05$).

of 50−100 μg/mL could arrest the HT-29 and COLO 320DM cancer cell lines at the G_0/G_1 phase.

To test the protein expressions of the PTE-induced G_1 phase block on the CRC cells, the levels of cell-cycle-regulating proteins cyclin D1, cyclin E, cyclin A, cyclin B, p21, and p27 were determined by immunoblotting. The levels of β-actin served as an internal control. As shown in Figure 3, the cyclin D1 level gradually decreased in both PTE-treated cells. The cyclin E, A, and B levels gradually decreased in the PTE-treated cells of the COLO 320 DM but not the HT-29. Moreover, the levels of p21 and p27 gradually increased only in the HT-29 with increased concentrations. The level of p21 increased 3.85-fold significantly in the PTE-treated HT-29 with PTE > 100 μg/mL. PTE blocked the progression of the cell cycle at the G1 phase by inducing p21 and p27 expression and, in turn, downregulating cyclin D1 expression in the HT-29 cell. However, the cell arresting properties of PTE in COLO

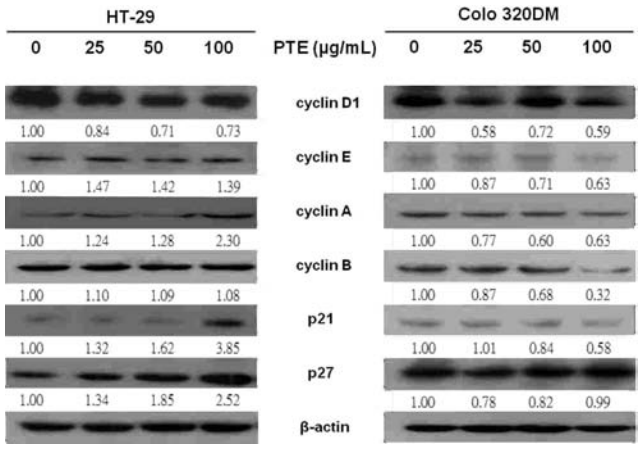

Figure 3. Immunoblots of cell cycle-linked proteins in the PTE-treated CRC cells. Cells were treated with increasing concentrations of PTE as indicated, then incubated at 37 °C for 24 h. Cell protein lysates from (A) HT-29 and (B) COLO 320 DM cells were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted to show various cell cycle regulators, with beta-actin levels as the loading control. Protein levels were quantified using ImageJ software according to the density of each band on the immunoblotting image, normalized to the reference band (β-actin), and presented as the folds of untreated control.

320DM were through downregulating cyclin E, A, and B expression. Although different regulating proteins were involved in the HT-29 and COLO 320DM cancer cell lines, the G_0/G_1 phase arrest was related to the downregulation of cyclin D1 and cyclin E and the upregulation of p21 and p27 expression.

PTE-Induced Apoptotic Death of CRC Cells. Phosphatidylserine translocation was assessed to determine the apoptosis of the PTE-treated CRC cells by staining with FITC-conjugated annexin V. Annexin V positive cells increased in a dose-dependent manner in the HT-29 and COLO 320DM cells (Figure 4A,B). The HT-29 and COLO 320DM showed significantly more apoptotic cells at 100 μg/mL ($P < 0.05$). Within the first 24 h, PTE induced a 14.11% and 21.98% degree of apoptosis of the HT-29 cells and COLO 320DM exposed to 100 μg/mL. As compared to the control, PTE at a concentration of 100 μg/mL induced two times more apoptosis in HT-29 and 1.3 times in COLO 320DM. The balance between survival and apoptosis often tips toward the former in cancer cells. Tea polyphenol treatment has been shown to induce apoptosis in colon cancer cell lines.[27,30]

Bcl-2 protein has been associated with apoptosis inhibition, whereas the expression of Bax has been associated with apoptosis induction.[31,32] The Bax/Bcl-2 ratio is important in apoptosis.[33] Although Bcl-2 protein expression was not found in the HT-29 cells, the Bax protein expression significantly increased (Figure 5). PTE-treated COLO 320DM showed a slightly decreased Bcl-2 but an increased Bax. The requirement of caspase 3 as an executor in tea polyphenol-induced apoptosis was demonstrated both directly and indirectly. Cleaved-caspase 3 levels in the PTE-treated HT-29 gradually increased (Figure 5). The PARP level also decreased in the HT-29, and the cleaved PARP increased more than 25 μg/mL in these two cell lines, closely correlating to cellular apoptosis (Figure 5).

Apoptosis induction is another possible mechanism of PTE on CRC cells. In the present study, we showed that PTE induced apoptosis significantly in the HT-29 and COLO

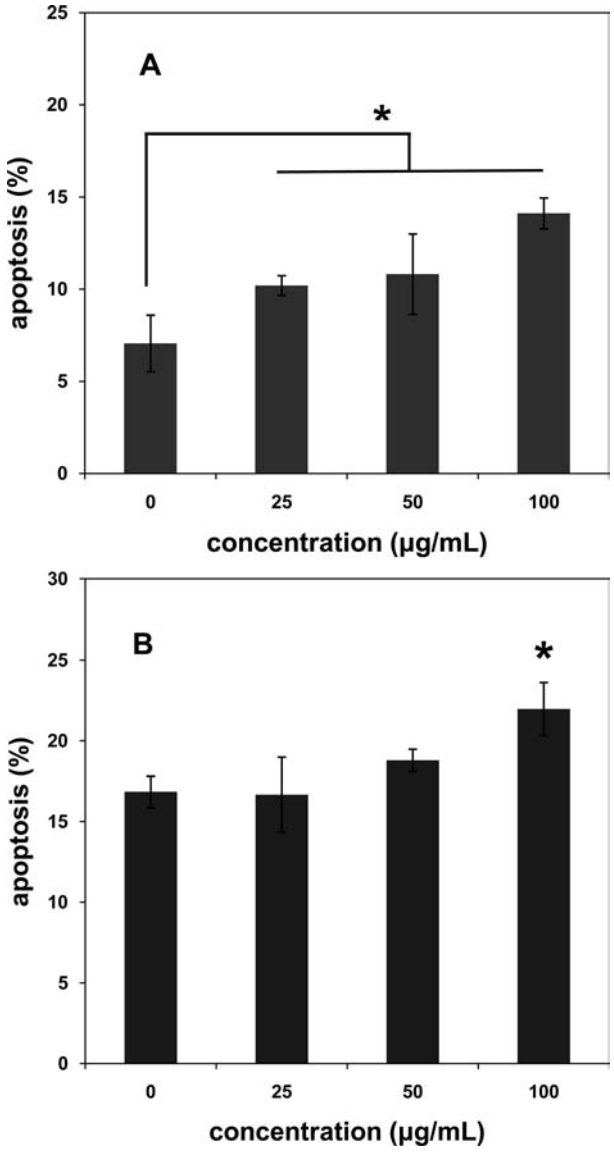

Figure 4. Sensitivity of apoptosis induced by PTE. HT29 cells (A) and COLO 320DM cells (B) were treated with increasing concentrations of PTE as indicated, then incubated at 37 °C for 24 h. The treated cells were then suspended and stained with annexin V, conjugated with FITC, and analyzed by flow cytometry. The data reported are the averages of three independent experiments and are expressed as means ± SD; *P < 0.05.

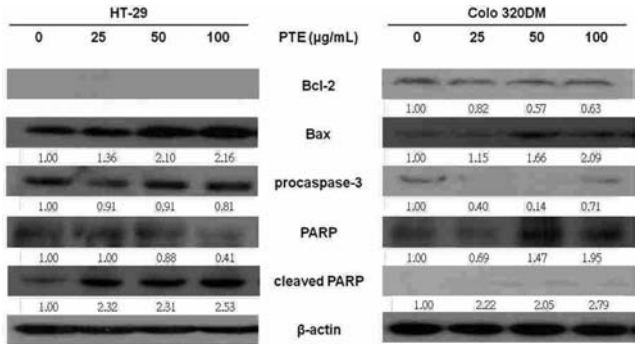

Figure 5. Immunoblots of the apoptotic-linked proteins in the PTE-treated CRC cells. Cells were treated with increasing concentrations of PTE as indicated, then incubated at 37 °C for 24 h. Cell protein lysates from (A) HT-29 and (B) COLO 320 DM cells were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted to show various apoptotic regulators, with beta-actin levels as the loading control. Protein levels were quantified using ImageJ software according to the density of each band on the immunoblotting image, normalized to the reference band (β-actin), and presented as the folds of untreated control.

320DM cells at concentrations of 25−100 μg/mL. PTE-induced apoptosis was mediated by the activation of caspase 3. Many reports have suggested that the polyphenolic-induced apoptosis in cancer cells was mediated by the activation of caspase 3, and the increasing cleavage fragments of its substrate PARP were a useful marker to indicate caspase 3 activity.[34−36] Although PTE treatment only slightly decreased the protein levels of pro-caspase 3 in the COLO 320DM cells, PARP was elevated and closely correlated with apoptotic induction. These results indicated that PTE-induced apoptosis in the HT-29 and COLO 320DM cells was mediated by caspase 3 activation. PTE-induced caspase 3 activation and apoptosis may operate through the Bcl-2 family of proteins. In our results, although PTE influenced different changes in the Bax and Bcl-2 expression levels in the HT-29 and COLO 320DM, the Bax/Bcl-2 ratio increased in both cell lines, and the ratios were closely correlated with apoptosis. These results underline the importance of the Bax/Bcl-2 ratio in cancer cell life and death.[32,37] Together, these results demonstrate that PTE-induced apoptosis in some CRC cells is mainly due to an increased Bax/Bcl-2 ratio and the concomitant caspase 3 activation.

The inhibitory effect of PTE on the proliferation of colorectal carcinoma cells may involve many cellular mechanisms, such as cell cycle arrest, the induction of apoptosis, and necrosis. Our investigation of the cell cycle distribution of PTE-treated HT-29 and COLO 320DM cells revealed that the cell cycle was arrested in the G_0/G_1 phase in both cell lines; however, different cell cycle regulating proteins were involved. Similarly, different representative apoptosis-related proteins induced apoptosis in HT-29 and COLO 320DM cells. We showed that PTE also induced necrosis in the CT-26 cell (data was not shown). Complicated composition and the abundant anthocyanidins, anthocyanins, and catechins of PTE were responsible for the prominent anticancer activity, but it was also because this complexity affected the signal pathways and cellular mechanisms in the different cell lines.

This research was conducted using a cellular model, which posed a limitation to explaining the conclusions about the role of the anthocyanins in PTE on anticancer activity. In the future, further studies are needed to identify the anthocyanins and compare them with a combination of pure anthocyanins.

This is the first study to show that PTE could inhibit proliferation in both the HT-29 and COLO 320DM colorectal cancer cell lines through signal pathways in a dose-dependent manner. The cellular mechanism responsible for the inhibitory effect of PTE on two colorectal cancer cells was mainly cell cycle arrest in the G_0/G_1 phase. The accumulation of p21 and p27 and the decrease of cyclin D and cyclin E in the PTE treatment of cells may be attributed to the molecular mechanisms that lead cells into G_0/G_1 phase arrest. Apoptosis may be another cellular mechanism responsible for the proliferation inhibition by PTE. These results suggest that the pathway of PTE-induced apoptosis involves the induction of Bax and the concomitant cleavage of caspase 3 and PARP.

The purple-shoot teas are a potential raw material for the production of an anthocyanins- and anthocyanidins-rich health drink since they are highly soluble in water with its bioactivity. They also have an added advantage over other polyphenols in that they have been found to provide the additional health benefit of colorectal cancer chemoprevention.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Ju, J. H.; Chang, S. C.; Wang, H. S.; Yang, S. H.; Jiang, J. K.; Chen, W. C.; Lin, T. C.; Hsu, H.; Wang, F. M.; Lin, J. K. Changes in disease pattern and treatment outcome of colorectal cancer: a review of 5,474 cases in 20 years. *Int. J. Colorectal Dis.* **2007**, *22*, 855−862.

(2) Lin, C.-C.; Lu, M.-J.; Chen, S.-J.; Ho, S.-C. Heavy fermentation impacts NO-suppressing activity of tea in LPS-activated RAW 264.7 macrophages. *Food Chem.* **2006**, *98*, 483−489.

(3) Yang, C. S.; Lambert, J. D.; Ju, J.; Lu, G.; Sang, S. Tea and cancer prevention: molecular mechanisms and human relevance. *Toxicol. Appl. Pharmacol.* **2007**, *224*, 265−273.

(4) Lin, J.-K.; Liang, Y.-C.; Lin-Shiau, S. Y. Cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade. *Biochem. Pharmacol.* **1999**, *58*, 911−915.

(5) Shimizu, M.; Weinstein, I. B. Modulation of signal transduction by tea catechins and related phytochemicals. *Mutat. Res.* **2005**, *591*, 147−160.

(6) Hou, Z.; Lambert, J. D.; Chin, K.-W.; Yang, C. S. Effects of tea polyphenols on signal transduction pathways related to cancer chemoprevention. *Mutat. Res.* **2004**, *555*, 3−19.

(7) Yang, C. S.; Wang, H.; Li, G. X.; Yang, Z.; Guan, F.; Jin, H. Cancer prevention by tea: evidence from laboratory studies. *Pharmacol. Res.* **2011**, *64*, 113−122.

(8) Kuo, P.-L.; Hsu, Y.-L.; Lin, T.-C.; Lin, C.-C. The antiproliferative activity of prodelphinidin B-2,3′-*O*-gallate from green tea leaf is through cell cycle arrest and Fas-mediated apoptotic pathway in A549 cells. *Food Chem. Toxicol.* **2005**, *43*, 315−323.

(9) Friedman, M.; Mackey, B. E.; Kim, H.; Lee, I.; Lee, K.; Lee, S.; Kozukue, E.; Kozukue, N. Structure−activity relationships of tea compounds against human cancer cells. *J. Agric. Food Chem.* **2007**, *55*, 243−253.

(10) Faria, A.; Oliveira, J.; Neves, P.; Gameiro, P.; Santos-Buelga, C. Antioxidant properties of prepared blueberry (*Vaccinium myrtillus*) extracts. *J. Agric. Food Chem.* **2005**, *53*, 6896−6902.

(11) Kalbasi, A.; Cisneros-Zevallos, L. Fractionation of monomeric and polymeric anthocyanins from concord grape (*Vitis labrusca* L.) juice by membrane ultrafiltration. *J. Agric. Food Chem.* **2007**, *55*, 7036−7042.

(12) Jacob, J. K.; Hakimuddin, F.; Paliyath, G.; Fisher, H. Antioxidant and antiproliferative activity of polyphenols in novel high-polyphenol grape lines. *Food Res. Int.* **2008**, *41*, 419−428.

(13) Wang, L.-S.; Stoner, G. D. Anthocyanins and their role in cancer prevention. *Cancer Lett.* **2008**, *269*, 281−290.

(14) Kerio, L. C.; Wachira, F. N.; Wanyoko, J. K.; Rotich, M. K. Characterization of anthocyanins in Kenyan teas: extraction and identification. *Food Chem.* **2012**, *131*, 31−38.

(15) Saito, T.; Honma, D.; Tagashira, M.; Kanda, T.; Nesumi, A.; Maeda-Yamamoto, M. Anthocyanins from new red leaf tea 'Sunrouge'. *J. Agric. Food Chem.* **2011**, *59*, 4779−4782.

(16) Zhao, C.; Giusti, M. M.; Malik, M.; Moyer, M. P.; Magnuson, B. A. Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth. *J. Agric. Food Chem.* **2004**, *20*, 6122−6128.

(17) Julkunen-Titti, R. Phenolic constituents in the leaves of northern willows: methods for the analysis of certain phenolics. *J. Agric. Food Chem.* **1985**, *33*, 213−217.

(18) Zhishen, J.; Mengcheng, T.; Jianming, W. The determination of flavonoids contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* **1999**, *64*, 555−559.

(19) Fuleki, Y.; Francis, F. J. Quantitative methods for anthocyanidins. 1. Extraction and determination of total anthocyanin in cranberries. *J. Food Sci.* **1968**, *33*, 72−77.

(20) Luximon-Ramma, A.; Bahorun, T.; Soobrattee, M. A.; Aruoma, O. I. Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in extracts of Cassia fistula. *J. Agric. Food Chem.* **2002**, *50*, 5042−5047.

(21) Hsu, C.-P.; Lin, Y.-H.; Wang, S.-C.; Zhou, S.-P.; Chung, Y.-C.; Lin, C.-C. *Longan* flower extract inhibits the growth of colorectal carcinoma. *Nutr. Cancer* **2010**, *62*, 229−36.

(22) Chung, Y. C.; Lin, C.-C.; Chou, C.-C.; Hsu, C. P. The effect of *Longan* seed polyphenols on colorectal carcinoma cells. *Eur. J. Clin. Invest.* **2010**, *40*, 713−721.

(23) Hsu, C.-P.; Lin, Y.-H.; Chou, C.-C.; Zhou, S.-P.; Hsu, Y.-C.; Liu, C.-L. Mechanisms of *grape* seed procyanidin-induced apoptosis in colorectal carcinoma cells. *Anticancer Res.* **2009**, *29*, 283−289.

(24) Xiao, L.-Z.; Su, X.-Q.; Li, Q.; Liu, Z.-H.; Zhang, D.-M.; Luo, H.-H. Biochemical components in the shoot of purple bud tea varieties. *J. Fujian Agric. For. Uni. (Natural Science Edition)* **2009**, *38*, 30−33 (in Chinese)..

(25) Pietrini, F.; Iannelli, M. A.; Massacci, A. Anthocyanin accumulation in the illuminated surface of maize leaves enhances protection from photo-inhibitory risks at low temperature, without further limitation to photosynthesis. *Plant Cell Environ.* **2002**, *25*, 1251−1259.

(26) Malik, M.; Zhao, C.; Schoene, N.; Guisti, M. M.; Moyer, M. P.; Magnuson, B. A. Anthocyanin-rich extract from *Aronia meloncarpa* E. induces a cell cycle block in colon cancer but not normal colonic cells. *Nutr. Cancer* **2003**, *46*, 186−196.

(27) Tan, X.; Hu, D.; Li, S.; Han, Y.; Zhang, Y.; Zhou, D. Differences of four catechins in cell cycle arrest and induction of apoptosis in LoVo cells. *Cancer Lett.* **2000**, *158*, 1−6.

(28) Way, T.-R.; Lin, H.-Y.; Kuo, D.-H.; Tsai, S.-J.; Shieh, J.-C.; Wu, J.-C.; Lee, M.-R.; Lin, J.-K. Pu-erh tea attenuates hyperlipogenesis and induces hepatoma cells growth arrest through activating AMP-activated protein kinase (AMPK) in human HepG2 cells. *J. Agric. Food Chem.* **2009**, *57*, 5257−5264.

(29) Prasad, S.; Kaur, J.; Roy, P.; Kalra, N.; Shukla, Y. Theaflavins induce G2/M arrest by modulating expression of $p21^{waf1/cip1}$, cdc25C and cyclin B in human prostate carcinoma PC-3 cells. *Life Sci.* **2007**, *81*, 1323−1331.

(30) Hwang, J.-T.; Ha, J.; Park, I.-J.; Lee, S.-K.; Baik, H. W.; Kim, Y. M.; Park, O. J. Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway. *Cancer Lett.* **2007**, *247*, 115−121.

(31) Oltvai, Z. N.; Milliman, C. L.; Korsmeyer, S. J. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **1993**, *74*, 609−619.

(32) Reed, J. C. Balancing cell life and death: bax, apoptosis, and breast cancer. *J. Clin. Invest.* **1996**, *97*, 2403−2404.

(33) Chresta, C. M.; Masters, J. R.; Hickman, J. A. Hypersensitivity of human testicular tumors to etoposide-induced apoptosis is associated with functional p53 and a high Bax:Bcl-2 ratio. *Cancer Res.* **1996**, *56*, 1834−1841.

(34) Islam, S.; Islam, N.; Kermode, T.; Johnstone, B.; Mukhtar, H.; Moskowitz, R. W.; Goldberg, V. M.; Malemud, C. J.; Haqqi, T. M. Involvement of caspase-3 in epigallocatechin-3-gallate-mediated apoptosis of human chondrosarcoma cells. *Biochem. Biophys. Res. Commun.* **2000**, *270*, 793−797.

(35) Raza, H.; John, A. Green tea polyphenol epigallocatechin-3-gallate differentially modulates oxidative stress in PC12 cell compartments. *Toxicol. Appl. Pharmacol.* **2005**, *207*, 212−220.

(36) Engelbrecht, A. M.; Mattheyse, M.; Ellis, B.; Loos, B.; Thomas, M.; Smith, R. Proanthocyanidin from *grape* seeds inactivates the PI3-

kinase/PKB pathway and induces apoptosis in a colon cancer cell line. *Cancer Lett.* **2007**, *258*, 144−153.

(37) Reed, J. C.; Miyashita, T.; Takayama, S.; Wang, H. G.; Sato, T.; Krajewski, S. BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. *J. Cell Biochem.* **1996**, *60*, 23−32.