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Anti-proliferation and radiation-sensitizing effect of an anthocyanidin- rich extract from purple-shoot tea on colon cancer cells

Anti-Proliferation and Radiation-Sensitizing Effect of an Anthocyanidin- Rich Extract from Purple-Shoot Tea on Colon Cancer Cells

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ABSTRACT

Teas are known to have several health promoting benefits due to polyphenol contents. Two varieties of tea cyanins, Purple-Shoot Tea (not registered) and TTES No. 12, were selected, dehydrated or prepared to green tea to evaluate the anti-proliferative and radiation-sensitizing effects on colon cancer cells. The tea cyanins were extracted by using three drying treatments which are freeze-dried, hot-air dried and green tea manufacturing. The composition analysis showed that the extracts of purple-shoot teas contained more polyphenolic, flavonoids, condensed tannins, anthocyanins and proanthocyanidins than TTES No. 12 under various tea processes. Lyophilized tea samples had better antioxidant capacities and reducing power than those of samples from hot-air dried and green tea manufacturing. Anti-proliferation results from HT-29, Colo 320DM and CT-26 cell lines showed that Purple-shoot tea had prominent inhibitory activities which might be correlated to its antioxidative properties. The cell cycle studies confirmed that Purple-shoot tea arrested colon cancer cell at G_0/G_1 phase. Tested tea samples had radiation-sensitizing effect on the CT-26 cell with 2Gy Co-60 irradiation. Higher anthocyanidin and anthocyanin contents in Purple-shoot tea may contribute to enhanced cytotoxicity and induction of apoptosis after irradiation. The radiation-sensitive effect of Purple-shoot tea on carcinoma cells suggested that nature compounds such as tea polyphenol can be utilized to reduce the dosage in radiotherapy in the future.

Key words: anthocyanidin, anthocyanin, tea, proliferation, radiation-sensitive

INTRODUCTION

The incidence of digest tract-related cancer is highly affected by diet, which suggests that cancer is suitable target disease for the dietary agents. In Taiwan, the culture food was gradually switched to western style diet on the past three decades. Colon cancer leaped forward to the third highest death rate of cancers. Surgery is the common method that was the primary treatment for malignant lesions. Other strategies of colon cancer may be combined radiotherapy or chemotherapy. Radiotherapy killed most tumor cells by inducing DNA damage, yet some uncomfortable side effects can not be avoided. Natural products have become another popular alternative for the prevention or treatment of cancer. Moreover, a large number of natural compounds have shown cytotoxic effects in different cancer models either alone or together with irradiation[(1)].

Tea (*Camellia sinensis*) with high levels of catechins is the most widely consumed popular beverage worldwide and regarded as health drink[(2)]. Tea catechins can scavenge reactive oxygen species, and thereby directly or indirectly exhibit a broad spectrum of biological, pharmacological activity against proliferation of carcinoma via modulate important cellular signaling processes. Three major commercial tea varieties such as Green, Oolong and Black teas are usually consumed, but most research projects demonstrating the anti-mutagenic and anti-carcinogenic effects of tea have been conducted with water extract of green tea, or a polyphenolic fraction isolated from green tea. The most active compound is (−)-epigallocatechin-3-gallate, which is also the major constituent of green tea. However, the manufacturing method to which composition contributes the anti-carcinogenic activity of tea has seldom been examined for the same tea variety.

Special tea varieties with purple or red colored buds and leaves, have been shown that contain high contents of anthocyanidins and anthocyanins[(3,4)]. Interests in anthocyanins and anthocyanidins contents have recently

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increasing due to the applicable health beneficial properties and pharmacological activities, which include antioxidant and antiproliferate properties. One recently bred cultivar of tea clones in Taiwan, purple-shoot tea clone, had not been investigated though it is believed that they should be rich in anthocyanidins and anthocyanins. It was also expected that the contents of anthocyanidins and anthocyanins in addition to the catechins would contribute to more effective activities.

The purpose of this study was to investigate anti-proliferation effects of various processed purple-shoot tea on the colorectal cell lines. Two colorectal carcinoma (CRC) cell lines (Colo 320DM and HT-29) and rat small intestine epithelial cell line (IEC-6) were treated with tea samples and assessed for viability. The presence of purple-shoot tea during irradiation amplifies radiation effects by inducing toxic reactions of free radicals was also investigated.

MATERIALS AND METHODS

I. *Materials and Cell Lines*

Folin-Ciocalteu reagent and standard 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). Annexin V conjugated with fluorescein isothiocyanate (FITC) and propidium iodide was from BD Co. (USA). All other chemicals were of analytical purity grade.

Human CRC cell lines HT-29, Colo 320DM and Rat small intestine epithelial cell IEC-6 were obtained from the Bioresource Collection and Research Center (Taiwan) and mouse colorectal carcinoma cell lines (CT-26) were purchased from American Type Culture Collection (ATCC). The cells were incubated at 37℃ in a 95% air/5% CO_2 and water-saturated atmosphere.

II. *Sample Preparation*

Purple-shoot tea and TTES No.12 tea samples were kindly provided by the Tea Research and Extension Station (Taiwan). Young tender shoots comprising of two unfolded younger leaves with a bud were hand harvested. Fresh tea leaves were freeze dried (F), hot-air dry (H) or prepare to green tea (G) until a constant weight was achieved. All of tea was milled into powder by using a grinder. The powder samples were extracted twice with a 50-fold volume of methanol for 60 min while shaken vigorously. After the samples were filtered with Whatman No.1 filter papers, the filtrates were evaporated under a vacuum below 50℃ by using a rotary evaporator. The extracts of Purple-Shoot Tea and TTES No.12 tea were defined as PT and TT.

III. *Characterization of Phytochemicals*

The total phenolics (mg gallic acid eq./g), total flavonoids (mg catechin eq./g), condensed tannin (mg catechin eq./g), anthocyanin (μg cyanidin-3-glucoside eq./g), anthocyanidin (mg cyanidin eq./g) presenting in the tea extract were determined by colorimetric assay as reported previously[5]. Catechins and anthocyanidin were performed by HPLC with an ODS HYPERSIL (Thermo scientific) reverse phase column (25cm × 0.46cm i.d., 5 μ) The mobile phase of 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 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. The identifications of catechins and flavonoids were determined by HPLC according to retention times obtained from authentic standards run at identical conditions.

IV. *Irradiation Procedure*

IEC-6 and CT-26 cells dispersed in 25 cm^2 dishes and 200 μg/mL samples were added. Cells were irradiated after 4 h incubation of PTF, with a Co-60 unit (Institute of Nuclear Energy Research) at a dose rate of 10 Gy/min. The samples were re-incubated for 24 h and 48 h.

V. *Cell Proliferation Assay*

Two CRC cell lines (HT-29 and Colo320DM) and rat small intestine epithelial cell line (IEC-6) were plated at 10^5 cells in 60-mm tissue culture dishes. After 18 h of culture, cells were treated with different concentrations of DMSO-dissolved PTF (0, 25, 50, 100, 200 or 400 μg mL-1). At 24 h and 48 h, 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. The cell cycle distribution of PTF-treated cells was measured by the DNA contents in each cell using flow cytometry. Colony numbers were conuted under visual observation.

RESULTS AND DISCUSSION

I. *Polyphenols Content*

Total phenolic content, total flavonoids, condensed tannin, anthocyanins, anthocyanidins of various tea s are shown in Table 1. On comparing the same amounts of tea extract, PTs contained higher concentration of phenolic compounds, and rich in flavonoids, condensed tannins, anthocyanin and anthocyanidin. Content of abundant anthocyanin in PT was observed as expected, and

surpasses 135 times than TT. Moreover, freeze dried treatment (F) appeared to retain higher anthocyanidins and anthocyanin than H and G significantly.

Table 1. The contents of total phenolics, total flavonoids and condensed tannin in various extracts of tea

Sample	Total phenols (mg/g)	Total flavonoids (mg/g)	Condensed tannins (mg/g)	Proanthocyanidin (mg/g)	Anthocyanin (μg/g)
PTG	179.7[b]	58.7[b]	156.0[a]	73.4[a]	3233.6[a]
PTF	200.3[a]	61.7[a]	160.4[a]	77.6[a]	3731.6[a]
PTH	155.3[b]	47.1[c]	72.7[b]	47.9[b]	2263.2[b]
TTG	159.7[b]	44.1[c]	84.0[b]	25.4[c]	14.9[c]
TTF	157.9[b]	48.2[c]	65.2[c]	22.1[c]	27.6[c]
TTH	132.9[c]	39.6[d]	61.9[c]	23.7[c]	14.9[c]

Means in the same column for each tea extract with different superscripts are significantly different ($p < 0.05$).

The major catechins in the both tea leaf include: EGCG, EGC, ECG and EC with the most abundant in tea was being EGCG (Table 2). Similar results were also found for catechins for PTF. Similar contents of catechins among three dehydration treatments were observed. Generally, the contents of catechins in PTF were higher than those of PTG and PTH. PT contained four anthocyanidins include: delphindin, cyanidin, malvindin, peonidin which have been tentatively identified by HPLC.

Table 2. The contents of catechins in various extracts of tea

Sample	gallic acid	EGC	EC	EGCG	ECG
PTG	ND	107.92[b]	22.62[b]	275.30[b]	64.17[a]
PTF	0.65[b]	163.06[a]	48.88[a]	319.82[a]	72.32[a]
PTH	5.3[a]	33.98[f]	22.07[b]	183.36[d]	75.60[a]
TTG	ND	88.60[c]	15.48[c]	222.95[c]	28.25[b]
TTF	0.61[b]	75.70[d]	10.13[d]	243.28[c]	24.29[b]
TTH	5.41[a]	40.40[e]	4.89[e]	228.29[c]	36.10[b]

All data were present in mg/g.

II. *Antiproliferative Activities of Teas*

The cell viability decreased in all tea-treated cell after 24 h and 48 h compared with untreated cells (Table 3, 4). However, these results demonstrated that purple-shoot tea exhibited higher inhibitory effects on proliferation of these two CRC cell lines. The IC_{50} values of PTF on HT-29 (Table 3) and Colo 320DM (Table 4) were significantly lower than those of others. Inhibitory efficacy of purple-shoot tea was higher than regular tea which preparing to Green Tea or Oolong Tea in Taiwan. Similar sensitivities of these two carcinoma cell lines to PTF in this assay were observed, however, HT-29 cells exhibited higher sensitivity to PTF than Colo 320DM cells.

Although no statistical difference was showed for the levels of total phenolic, total flavonoids in both PT and TT (Table 1), PT was still a stronger inhibitor on carcinoma cell among the tea extracts. These findings suggested that the anti-proliferation activity of PT might not only be related in a simple manner to the amount of phenolic compounds present. Proanthocyanidins and anthocyanin in PT, not heat stable, might play important roles in anti-proliferation activities. Light dehydration method was recommended whenever PT is prepared for developing novel health food.

Table 3. Antiproliferative activities of various tea extracts in HT-29 cell line

Sample	IC_{50} (μg/mL)	
	24 hr	48 hr
PTG	57.3 ± 13.4[b]	67.3 ± 13.4[a]
PTF	55.2 ± 10.8[b]	23.8 ± 6.1[b]
PTH	102.6 ± 12.1[a]	91.6 ± 1.6[a]
TTG	282.4 ± 21.8[a]	172.8 ± 2.8[a]
TTF	262.5 ± 13.5[a]	142.6 ± 5.2[a]
TTH	295.1 ± 10.2[a]	173.6 ± 13.3[a]

Table 4. Antiproliferative activities of various tea extracts determined in Colo 320DM cell line

Sample	IC_{50} (μg/mL)	
	24 hr	48 hr
PTG	83.23 ± 6.88[b]	41.60 ± 7.93[a]
PTF	64.90 ± 7.13[c]	22.42 ± 2.16[b]
PTH	127.07 ± 12.0[a]	30.97 ± 3.84[a]
TTG	132.50 ± 20.3[b]	74.51±3.54[a]
TTF	132.29 ± 31.0[b]	35.14 ± 4.52[b]
TTH	312.99 ± 4.46[a]	109.5 ± 8.77[a]

III. *Radiation-Sensitizing Activities of Teas*

The radiation-sensitizing effect of purple-shoot tea on the colorectal cell viability after Co-60 irradiation was investigated. Necrosis was determined by flow cytomerty

after 24 h. Tea-treated cells had minor increased amounts of necrosis cells (up to 12 - 25%) after irradiated for 24 h in both treated and untreated cells (Figure 1). The cytotoxicity effects resulted from the combination of teas and irradiation on IEC-6 were in the order as PTF > TTF > BTF. PTF induced significant necrosis in CT-26 cells after irradiated for 24 h. This was due to synergistic effect of PT and irradiation.

Late effect was performed by pretreating cells with of samples (200 μg/mL) for 4 h followed by irradiation treatments. Cells were cultured 7 days, fixed, and stained with Giemsa stain. From results of clonogenic cell survival assay, there was a complete cytotoxicity when PTF-treated or combined TTF with 2 Gy irradiation (Table 5). PTF exhibited prominent anti-proliferation activity without irradiation. The results demonstrated that 200 μg/mL concentration of PTF might too high for acting as a radiation-sensitizing agent in this system. Nevertheless, synergistic effect of 200 μg/mL for TTF and 2 Gy irradiation exhibited higher cytotoxicity on CT-6 than IEC-6. Further investigations are required to fully explore the detailed mechanisms involved.

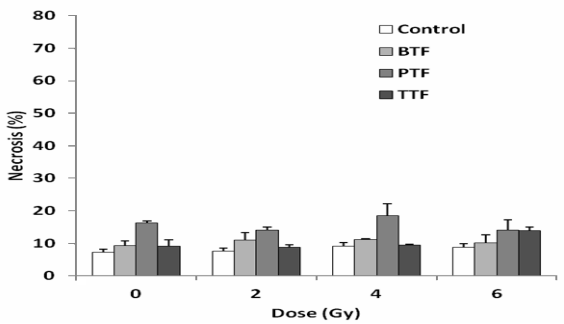

Figure 1. Acute effects that after irradiation on the treated IEC-6 cell.

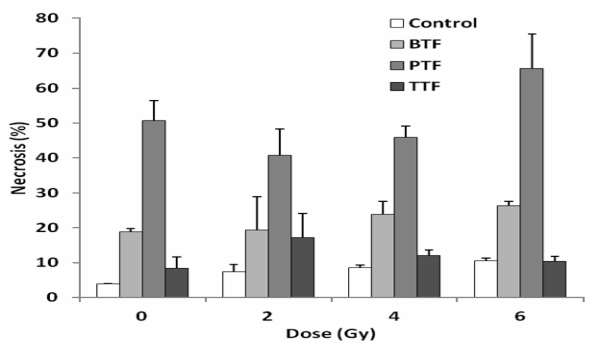

Figure 2. Acute effectsafter irradiation on the treated CT-26 cell.

CONCLUSIONS

Since total phenols are responsible for a wide spectrum of biological activities, the greater quantity of total phenols in PT indicates it possesses greater activity than TT. Other than catechins and flavonoids, the anti-proliferation and radiation-sensitizing of PT are attributed to the relationship between anthocyanidins and anthocyanin and the corresponding activity. Our results suggested that freeze-dried or prepared to green tea processing could retain more proanthocyanidins and anthocyanins, and appeared to be responsible for the function for anti-proliferation activity and radiationn-sensitizing effect of purple-shoot tea.

Table 5. Effect of tea extract on clonogenic fraction assay by irradiation

	IEC-6		CT-26	
	0 Gy	2 Gy	0 Gy	2 Gy
Control	100 ± 0.0	39.1 ± 20.1	100.0 ± 0.0	46.0 ± 24.9
PTF	1.5 ± 1.7	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
TTF	88.2 ± 25.0	53.0 ± 12.7	20.5 ± 12.8	0.0 ± 0.0

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