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Factors Affecting the Levels of Tea Polyphenols and Caffeine in Tea Leaves

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An isocratic HPLC procedure was developed for the simultaneous determination of caffeine and six catechins in tea samples. When 31 commercial teas extracted by boiling water or 75% ethanol were analyzed by HPLC, the levels of (−)-epigallocatechin 3-gallate (EGCG), and total catechins in teas were in the order green tea (old leaves) > green tea (young leaves) and oolong tea > black tea and pu-erh tea. Tea samples extracted by 75% ethanol could yield higher levels of EGCG and total catechins. The contents of caffeine and catechins also have been measured in fresh tea leaves from the Tea Experiment Station in Wen-Shan or Taitung; the old tea leaves contain less caffeine but more EGCG and total catechins than young ones. To compare caffeine and catechins in the same tea but manufactured by different fermentation processes, the level of caffeine in different manufactured teas was in the order black tea > oolong tea > green tea > fresh tea leaf, but the levels of EGCG and total catechins were in the order green tea > oolong tea > fresh tea leaf > black tea. In addition, six commercial tea extracts were used to test the biological functions including hydroxyl radical scavenging, nitric oxide suppressing, and apoptotic effects. The pu-erh tea extracts protected the plasmid DNA from damage by the Fenton reaction as well as the control at a concentration of 100 μg/mL. The nitric oxide suppressing effect of tea extracts was in the order pu-erh tea ≥ black tea > green tea > oolong tea. The induction of apoptosis by tea extract has been demonstrated by DNA fragmentation ladder and flow cytometry. It appeared that the ability of tea extracts to induce HL-60 cells apoptosis was in the order green tea > oolong > black tea > pu-erh tea. All tea extracts extracted by 75% ethanol have stronger biological functions than those extracted by boiling water.

KEYWORDS: Tea; tea leaves; catechins; tea polyphenols; caffeine; apoptosis; hydroxyl radical scavenging; nitric oxide suppressing

INTRODUCTION

Tea (*Camellia sinensis*) originated in southern China and is consumed by over two-thirds of the world's population. Tea has an attractive aroma, good taste, and health-promoting effects, and these benefits make it one of the most popular drinks in the world. As early as 3000 B.C., tea was used by the Chinese as a medicinal drink and as a beverage by the end of the sixth century. The medical use of tea was recorded in the ancient Chinese pharmacopoeia "Ben Cao Gang Mo" written by Shi-Zheng Li in the Ming dynasty (16th century). Because tea is an excellent beverage and has an effective pharmaceutical activity, tea plants are now widely cultivated in Southeast Asia including China, India, Japan, Taiwan, Sri Lanka, and Indonesia and in central African countries. Hundreds of teas are now produced and are generally classified into three major categories: nonfermented green tea, partially fermented oolong or paochong tea, and fully fermented black tea.

The composition of tea varies with species, season, age of the leaf (plucking position), climate, and horticultural practices (*1*). Green tea contains polyphenols, which include flavanols, flavadiols, flavonoids (*2*), and phenolic acids; these compounds may account for up 30% of the dry weight. The polyphenols are the most biologically active group of the tea components, especially certain catechins. The major tea catechins are (−)-epigallocatechin 3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (−)-epicatechin 3-gallate (ECG), and (+)-catechin (C), as depicted in **Figure 1**. In the manufacturing of black tea, the monomeric flavan-3-ols undergo polyphenol oxidase-dependent oxidative polymerization, which leads to the formation of bisflavanols, theaflavins, thearubigins, and other oligomers in the process commonly known as fermentation. Many biological functions of tea polyphenols have been studied (*1*, *3*, *4*). They include antioxidative activities (*5*−*7*), antimutagenic effects (*6*, *8*, *9*), anticarcinogenic effects (*6*, *8*−*10*), nitrosation inhibition (*11*, *12*), and inhibitory actions on the

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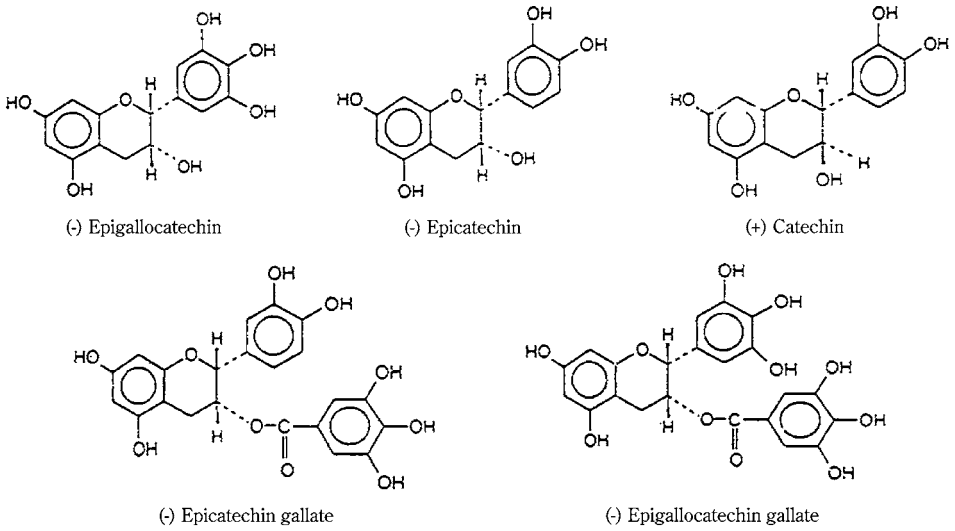

Figure 1. Structures of catechins.

Table 1. Serial Number, Name, and Place of Production of Tea Samples

serial no.[a]	name	place of production and purchase	remark
BT001	Lipton black tea	obtained from local market, Taiwan	United Li-Hwa Co., Taiwan
OC011	Xiao-Hong-Pao	Wuu-Yi Mountain, China	rock tea (high quality)
OC012	Gold Praise cinnamon	Wuu-Yi Mountain, China	rock tea (high quality)
OC013	Gold Praise narcissus	Wuu-Yi Mountain, Chian	rock tea (high quality)
OC014	rock tea	Wuu-Yi Mountain, China	rock tea (high quality)
OC015	Xiao-Hong-Pao	Wuu-Yi Mountain, China	rock tea (low quality)
OC016	Crowd narcissus	Wuu-Yi Mountain, China	rock tea (low quality)
OC017	Cinnamon King	Wuu-Yi Mountain, China	rock tea (low quality)
OC018	Da-Hong-Pao	Wuu-Yi Mountain, China	rock tea (low quality)
OC032	Xiao-Hong-Pao	Wuu-Yi Mountain, China	rock tea (high quality, spring tea)
OT013	1999 Dong-Ding oolong	Nan-Tou Lu-Gu, Taiwan	mountain tea (traditional oolong)
OT014	oolong tea	Ping-Lin, Taiwan	ground tea
OT015	Sunlight Tea Garden tea	Nan-Tou Jen-Ai, Taiwan	one bud, two youngest leaves
OT016	Shi-Zhuo-Zheng Lu Tea	Taiwan	traditional oolong tea
PC016	Dual Mountain pu-erh tea	Yun-Nan	tea cake, China
PC023	20-year-old jane pu-erh	Yun-Nan, China	purchased from Taiwan
PC024	Xiao-Huang-Yin	Yun-Nan, China	purchased from Taiwan
GC027	Zhu-Yeh-Ching	E-Mei Mountain, China	young leaf
GC028	Xian-Zhe-Zhu-Jian	E-Mei Mountain, China	young leaf
GC029	E-Mei-Shan Snow Bud	E-Mei Mountain, China	young leaf
GC048	longjing tea	Zhe-Jiang, China	young leaf mutation of white tea
GC053	green tea	China	young leaf
GC055	Huang-Shan Hair Peak	Huang Mountain, China	young leaf
GC056	Huang-Shan Hair Peak	Huang Mountain, China	young leaf
GC058	longjing tea	Zhe-Jiang University, China	young leaf
GC060	Lu-Shan cloud tea	Lu Mountain, China	old leaf, wild growth
GC061	Lu-Shan cloud tea	Lu Mountain, China	old leaf, cultivated in tea garden
GT001	longjing tea	Shan-Xia, Taiwan	old leaf
GJ002	decoct tea	Japan	old leaf
GJ003	decoct tea	Japan	old leaf
GJ013	Fancy Decoct Tea	Japan	old leaf

[a] The sample serial number was based on the tea group (B, black tea; O, oolong tea; P, pu-erh tea; G, green tea) and the place of production (C, China; T, Taiwan; J, Japan); the sample number was assigned arbitrarily on the basis of the order of collection. For example, BT001 means the black tea, from Taiwan, sample 001.

growth of tumor and immortalized cell but no effects on normal cells (*1*, *13*) in several systems.

Nitric oxide (NO) has a role in mediating macrophage cytotoxicity, regulating blood pressure, and neurotransmission. Molecular cloning and sequencing analysis have revealed that there are at least three main types of nitric oxide synthase (NOS) isoforms. Two Ca^{2+}/calmodulin-dependent isoforms are constitutively expressed in the endothelium of blood vessel and the neuron of the brain. The high-output isoform, inducible-NOS (iNOS), is expressed in various cell types following its transcriptional induction (*14*). Among the most important stimuli for induction of iNOS is bacterial endotoxic lipopolysaccharide (LPS) (*15*, *16*). However, high concentrations of NO and its derivatives, such as peroxynitrite and nitrogen dioxide, are found to play important roles in inflammation and in the multistage processes of carcinogenesis (*17*, *18*). Some studies have shown tea polyphenols suppression of LPS induced iNOS and generated NO by inhibition of nuclear factor-κB (NF-κB) (*4*, *19*, *20*).

Apoptosis is induced by a variety of stimuli, such as genotoxic compounds (*21*), tumor necrosis factor (*22*), Fas ligand (*23*), and various environmental stresses (*24*). Despite the diversity of apoptosis-inducing agents, numerous experiments indicate that signals leading to the activation of members of the intracellular cysteine protease family, for instance, the caspases, may play a pivotal role in the initiation and execution of apoptosis induced by various stimuli (*25*). To date, at least 10

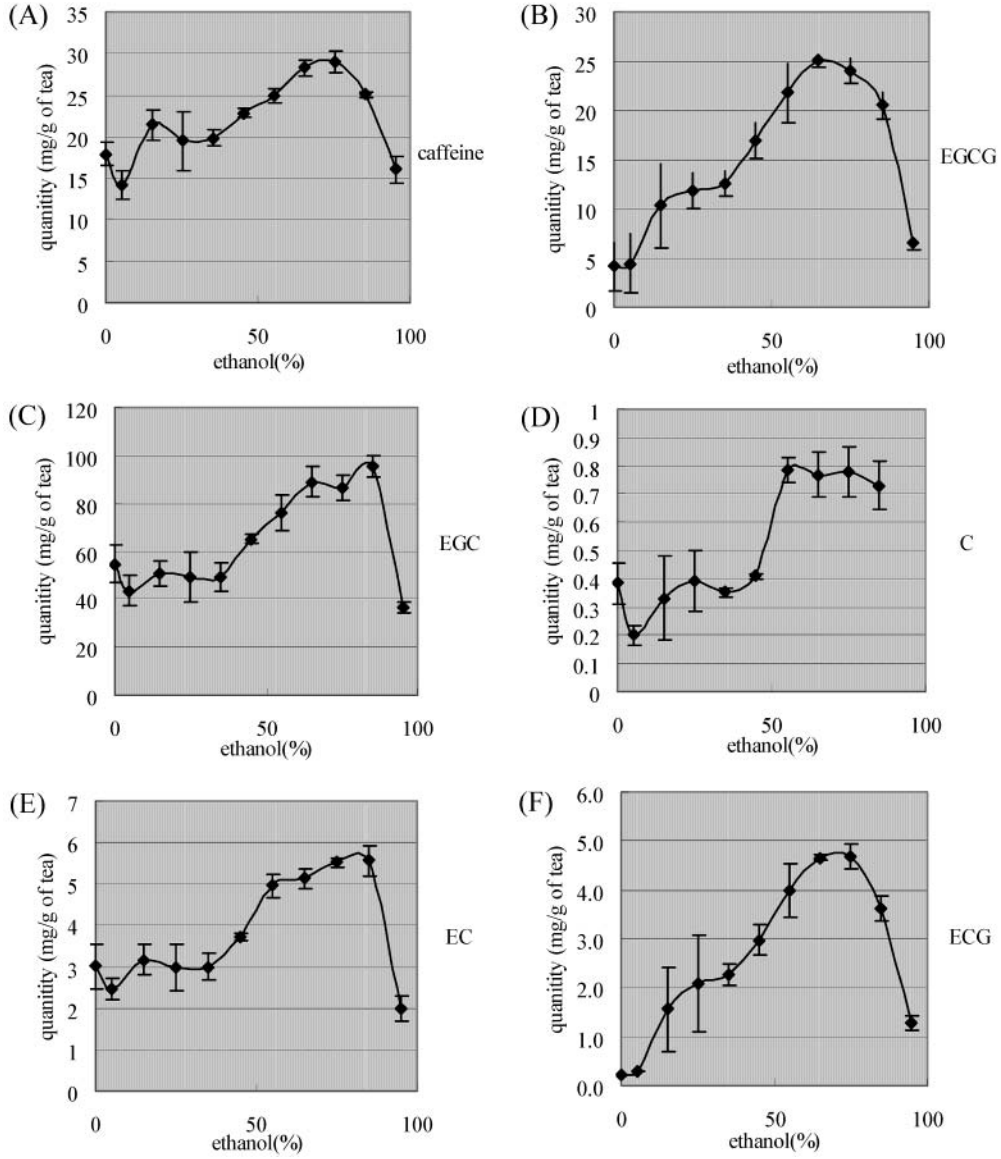

Figure 2. Analysis of caffeine and catechins in oolong tea (OT015) extracted by different percentages of ethanol. Each value represents the mean ± SE of three individual determinations.

distinct caspases in mammalian cells have been identified (*26*). Our previous studies demontrated that the tea polyphenols can induce apoptosis in a dose-dependent manner in U937 cells and HL-60 cells. The molecular mechanisms of the apoptotic effects induced by tea polyphenols were also evaluated in the studies (*27*, *28*).

In the present study, a simple and precise isocratic HPLC procedure was used to determine the composition of tea caffeine and catechins in various tea samples extracted by water and 75% ethanol. The study investigated the levels of tea caffeine and catechins in the samples of green teas, oolong teas, black teas, and pu-erh teas from China, Japan, and Taiwan. In addition, levels of tea caffeine and catechins in fresh tea leaves from the tea experiment station in Wen-Shan or Taitung were also investigated. This study was to compare the effect of different tea crude extracts on hydroxyl radical scavenging, nitric oxide suppression, and apoptosis.

MATERIALS AND METHODS

Chemicals and Reagents. Caffeine, (−)-epigallocatechin 3-gallate (EGCG), (−)-epigallocatechin (EGC), (+)-catechin (C), (−)-epicatechin (EC), (−)-epicatechin 3-gallate (ECG), (−)-gallocatechin 3-gallate (GCG), LPS (*Escherichia coli* O127:E8), sulfanilamide, dithiothreitol (DTT), *N*-1-naphthylethylenediamine dihydrochloride, and propidium iodide were purchased from Sigma Chemical Co. (St. Louis, MO). Agarose, ethidium bromide, dimethyl sulfoxide (DMSO), hydrogen peroxide, and ferrous sulfate were purchased from E. Merck Co. (Darmstadt, Germany). The plasmid pcDNA3, 1-E expression vector, was purchased from Invitrogen Life Technologies, Carlsbad, CA.

Tea and Fresh Tea Leaf Samples. Thirty-one commercial tea samples including green, oolong, black, and pu-erh teas from Taiwan, China, and Japan were analyzed. These tea samples were collected from the local markets (**Table 1**). The sample serial number was based on the tea group (B, black tea; O, oolong tea; P, pu-erh tea; G, green tea) and the place of production (C, China; T, Taiwan; J, Japan); the sample number was assigned arbitrarily on the basis of the order of collection. For example, BT001 means the black tea, from Taiwan, sample 001. Fresh tea leaves were plucked from the experiment station in Wen-Shan or Taitung and then dried at 70 °C overnight in an electric oven with rotating fan to keep the heat evenly distributed.

Preparation of Extracts from Tea and Fresh Leaves. Each of the dry tea leaves was steeped in boiling distilled water (100 mL) for 30 min. The infusion was filtered with 0.45 μm PVDF filter disk (Millipore, Bedford, MA). The filtrate was analyzed by the HPLC system as described below. The filtrate was dried under reducing pressure by rotavapor to give powdered crude extract and kept in a

Table 2. Polyphenol Composition of Various Teas in Water Extract[a]

	mg/g of tea						
sample	caffeine	EGCG	EGC	C	EC	ECG	total monomeric catechins
BT001	31.95 ± 0.91	2.42 ± 0.31	10.83 ± 1.03	ND	1.17 ± 0.12	1.93 ± 0.28	**16.35**
OC011	24.82 ± 2.15	7.85 ± 1.51	56.28 ± 6.50	2.47 ± 0.12	3.44 ± 0.44	0.96 ± 0.28	**71.00**
OC012	15.79 ± 0.45	3.33 ± 0.22	40.85 ± 4.12	1.92 ± 0.24	2.94 ± 0.17	0.59 ± 0.08	**49.63**
OC013	17.19 ± 1.29	2.81 ± 0.04	35.76 ± 5.87	0.78 ± 0.19	2.38 ± 0.46	0.24 ± 0.04	**41.97**
OC014	15.42 ± 0.62	3.69 ± 0.28	47.02 ± 2.40	1.78 ± 0.11	3.15 ± 0.28	0.49 ± 0.06	**56.13**
OC015	14.51 ± 0.86	0.88 ± 0.08	25.45 ± 2.45	2.05 ± 0.05	1.61 ± 0.12	0.43 ± 0.03	**30.42**
OC016	16.35 ± 1.04	1.72 ± 0.21	14.18 ± 4.27	1.33 ± 0.08	1.76 ± 0.27	0.21 ± 0.06	**19.20**
OC017	16.71 ± 0.47	2.11 ± 0.05	18.75 ± 0.11	0.45 ± 0.09	1.67 ± 0.35	0.30 ± 0.06	**23.28**
OC018	18.92 ± 1.24	3.47 ± 0.21	44.30 ± 0.12	0.85 ± 0.05	2.68 ± 0.39	0.57 ± 0.27	**51.87**
OC032	29.26 ± 2.60	13.15 ± 1.08	38.39 ± 5.90	1.41 ± 0.21	5.12 ± 0.28	3.16 ± 0.83	**61.23**
OT013	12.36 ± 2.56	3.20 ± 1.40	32.44 ± 5.69	0.43 ± 0.36	2.09 ± 0.49	0.44 ± 0.05	**38.60**
OT014	19.55 ± 2.46	10.49 ± 4.03	78.54 ± 9.73	0.48 ± 0.14	4.01 ± 0.54	1.19 ± 0.62	**94.71**
OT015	19.69 ± 2.13	5.38 ± 1.69	60.27 ± 8.43	0.37 ± 0.07	3.27 ± 0.48	0.62 ± 0.17	**69.91**
OT016	20.76 ± 2.83	7.76 ± 3.17	73.43 ± 13.4	0.50 ± 0.07	3.89 ± 0.84	1.06 ± 0.34	**86.64**
PC016	18.47 ± 1.23	ND	ND	ND	ND	ND	**ND**
PC023	26.40 ± 3.81	0.05 ± 0.10	ND	ND	1.47 ± 0.24	0.38 ± 0.10	**1.90**
PC024	30.46 ± 1.92	5.96 ± 0.67	13.82 ± 0.77	1.42 ± 0.11	4.87 ± 0.59	9.56 ± 1.03	**35.63**
GC027	29.32 ± 5.03	18.29 ± 0.24	22.19 ± 0.44	0.92 ± 0.02	2.32 ± 0.17	3.81 ± 0.39	**47.53**
GC028	29.36 ± 3.07	24.47 ± 0.86	26.29 ± 1.41	1.00 ± 0.04	2.54 ± 0.29	5.35 ± 0.92	**59.65**
GC029	26.28 ± 0.94	14.74 ± 2.72	37.20 ± 2.06	1.10 ± 0.13	3.70 ± 0.91	3.14 ± 0.39	**59.88**
GC048	28.68 ± 0.94	8.41 ± 0.73	8.03 ± 3.51	1.24 ± 0.55	1.22 ± 0.06	1.81 ± 0.57	**20.71**
GC053	23.81 ± 2.51	10.74 ± 0.83	7.63 ± 2.42	0.87 ± 0.30	1.79 ± 0.11	3.54 ± 0.28	**24.57**
GC055	25.30 ± 0.07	12.54 ± 0.76	33.67 ± 1.72	0.79 ± 0.04	2.61 ± 0.30	2.04 ± 0.44	**51.65**
GC056	22.73 ± 2.15	8.34 ± 0.44	19.32 ± 1.46	0.70 ± 0.05	1.96 ± 0.46	1.30 ± 0.12	**31.62**
GC058	29.89 ± 3.24	19.58 ± 0.54	30.75 ± 0.84	1.14 ± 0.04	4.04 ± 0.92	4.33 ± 0.33	**59.84**
GC060	27.62 ± 2.17	32.08 ± 2.25	83.83 ± 2.41	1.39 ± 0.15	6.37 ± 0.20	7.80 ± 0.72	**131.47**
GC061	33.44 ± 1.05	30.64 ± 1.18	40.11 ± 7.08	1.70 ± 0.88	3.93 ± 0.15	8.68 ± 1.69	**85.06**
GT001	31.92 ± 3.07	31.17 ± 2.59	67.31 ± 4.81	0.98 ± 0.09	4.14 ± 0.27	5.18 ± 0.57	**108.78**
GJ002	27.77 ± 1.28	21.19 ± 1.51	83.62 ± 1.89	0.64 ± 0.08	5.69 ± 0.13	3.45 ± 0.33	**114.59**
GJ003	26.35 ± 0.24	21.30 ± 0.61	82.44 ± 3.23	0.96 ± 0.34	6.05 ± 0.34	3.64 ± 0.25	**114.39**
GJ013	31.58 ± 0.94	19.58 ± 1.19	9.05 ± 0.40	2.13 ± 0.04	7.62± 0.19	3.46 ± 0.30	**41.84**

[a] Each of the tea leaves (1 g) was extracted by 100 mL of boiling water for 30 min. Each value represents the mean ± SE of five individual determinations. ND, not detectable.

refrigerator at −20 °C until use. In most experiments, the crude extract was dissolved in DMSO (50 mg/mL) and diluted to desired concentrations.

Reverse-Phase HPLC Analysis of Tea Polyphenols and Caffeine. The composition of caffeine and catechins in different teas and fresh leaves was determined by HPLC analysis using a Waters 600E system controller. The Waters 484 tunable absorbance detector was used to detect tea constituents at 280 nm, and all peaks were plotted and integrated by a Waters 745 data module. The HPLC method used a Cosmosil 5C18-MS packed column (5 μm, 4.6 mm × 250 mm, code 379-72). The tea extract was filtered through a 0.45 μm filter disk and then was injected into the column. The concentrations of caffeine and catechins working solutions were 100 μg/mL. Five hundred nanograms of each authentic standard compound (caffeine, EGCG, EGC, C, EC, GCG, and ECG) was injected. The mobile phase was methanol/doubly distilled water/formic acid (19.5:82.5:0.3, v/v/v) and was degassed by sonication (Branson 5200) and run by an isocratic elution at a flow rate of 1.0 mL/min.

Protection of Supercoiled DNA from Strand Breakage by Fenton Reaction. pcDNA-3 superhelix form plasmid DNA (200 ng) was incubated with 0.35% H_2O_2 and 50 μM ferrous sulfate in the presence or absence various concentrations of crude extract at 37 °C for 30 min. DNA relaxation to an open circular form was induced in the presence of hydroxyl radicals generated by H_2O_2 and Fe^{2+} (*29*). DNA was separated on 1% agarose gel and stained with ethidium bromide.

Cell Culture. RAW 264.7 cells, which were derived from murine macrophages, were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). RAW 264.7 cells were cultured in DMED (without phenol red) supplemented with 10% ednotoxin-free, heat-inactivated fetal calf serum (Gibco, Grand Island, NY). When the cells reached a density of (2−3) × 10^6 cells/mL, they were activated by incubation in medium containing *E. coli* O127:E8 LPS (50 ng/mL). Various test crude extracts dissolved in DMSO were added together with LPS at a final concentration of 50 μg/mL or the indicated concentration.

HL-60 (human promyelocytic leukemia) cells obtained from ATCC were grown in 90% RPMI 1640 and 10% fetal bovine serum (Gibco), supplemented with 2 mM glutamine. Medium was normally changed to phenol red-free RPMI 1640 before crude extract treatment.

Nitrite Determination. The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction (*30*).

Flow Cytometry. HL-60 cells (2 × 10^5) were cultured in 60 mm Petri dishes and incubated for 24 h. Then cells were harvested, washed with phosphate-buffered saline (PBS), resuspended in 200 μL of PBS, and fixed in 800 μL of iced 100% ethanol at −20 °C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at 37 °C for 30 min. Then 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide−DNA complex was quantitated after excitation of the fluorescent dye by FACSCalibur cytometry (Becton Dickenson, San Jose, CA).

RESULTS

HPLC Analysis of Tea Polyphenols and Caffeine in Teas. A mixture of authentic standard catechins as well as caffeine was analyzed by isocratic HPLC procedure as described under Materials and Methods. The results shown in **Figure 2** are OT015 extracted by different percentages of ethanol. More EGCG was detected in the sample extracted by 65−75%

Table 3. Polyphenol Composition of Various Teas in 75% Ethanol Extract[a]

	mg/g of tea						
sample	caffeine	EGCG	EGC	C	EC	ECG	total monomeric catechins
BT001	35.30 ± 0.38	4.14 ± 0.39	61.78 ± 5.29	ND	1.06 ± 0.09	3.77 ± 0.33	**70.75**
OC011	31.42 ± 0.95	30.68 ± 1.64	87.67 ± 6.62	3.03 ± 0.22	5.03 ± 0.38	5.10 ± 0.29	**131.51**
OC012	22.17 ± 1.45	11.75 ± 0.77	42.10 ± 5.91	2.32 ± 0.14	2.64 ± 0.41	2.17 ± 0.09	**60.98**
OC013	19.75 ± 1.31	7.15 ± 0.57	21.53 ± 1.24	1.20 ± 0.14	1.53 ± 0.48	1.25 ± 0.08	**32.66**
OC014	26.40 ± 0.99	14.67 ± 0.87	43.83 ± 1.42	2.08 ± 0.06	2.95 ± 0.69	2.90 ± 0.20	**66.43**
OC015	15.91 ± 1.45	8.73 ± 0.55	18.09 ± 0.53	2.58 ± 0.06	1.85 ± 0.31	1.61 ± 0.32	**32.86**
OC016	11.24 ± 0.37	2.86 ± 0.06	7.84 ± 0.20	2.39 ± 0.41	0.77 ± 0.22	0.60 ± 0.07	**14.46**
OC017	20.28 ± 0.85	9.06 ± 0.32	17.81 ± 0.14	0.67 ± 0.05	1.96 ± 0.30	1.88 ± 0.11	**31.38**
OC018	26.42 ± 0.43	17.27 ± 1.28	39.27 ± 1.79	0.98 ± 0.23	3.88 ± 0.39	3.76 ± 0.35	**65.16**
OC032	37.25 ± 0.73	28.10 ± 2.04	72.74 ± 8.11	2.58 ± 0.23	5.53 ± 0.33	9.22 ± 0.54	**118.17**
OT013	19.60 ± 1.93	21.23 ± 1.21	79.52 ± 6.50	0.82 ± 0.10	5.01 ± 0.27	3.43 ± 0.18	**110.01**
OT014	23.79 ± 1.27	27.74 ± 0.64	99.64 ± 5.43	0.61 ± 0.05	5.25 ± 0.20	4.23 ± 0.27	**137.47**
OT015	30.54 ± 2.07	27.11 ± 3.96	93.90 ± 10.1	0.76 ± 0.08	5.84 ± 0.43	5.14 ± 0.61	**132.75**
OT016	27.51 ± 1.51	29.23 ± 1.40	115.00 ± 7.00	0.84 ± 0.14	6.23 ± 0.21	4.75 ± 0.24	**156.05**
PC016	23.32 ± 0.88	ND	ND	ND	ND	ND	**ND**
PC023	31.25 ± 1.90	0.18 ± ×d40.05	ND	ND	0.89 ± 0.07	0.35 ± 0.14	**1.42**
PC024	26.90 ± 1.67	4.79 ± 0.75	5.78 ± 1.53	0.72 ± 0.15	2.58 ± 0.25	10.08 ± 0.84	**23.95**
GC027	27.25 ± 2.26	17.05 ± 0.05	10.29 ± 0.14	0.39 ± 0.02	1.26 ± 0.24	6.01 ± 2.26	**35.00**
GC028	33.29 ± 2.73	29.38 ± 2.12	20.88 ± 1.32	0.50 ± 0.03	1.94 ± 0.25	9.98 ± 0.91	**62.68**
GC029	23.29 ± 1.04	14.90 ± 2.55	28.06 ± 0.75	0.49 ± 0.01	2.49 ± 0.36	5.51 ± 2.17	**51.45**
GC048	33.64 ± 0.41	19.17 ± 1.03	15.34 ± 1.50	0.45 ± 0.16	1.36 ± 0.11	4.62 ± 0.21	**40.94**
GC053	39.33 ± 0.59	35.72 ± 1.48	13.13 ± 1.18	1.22 ± 0.16	3.14 ± 0.28	14.00 ± 0.93	**67.21**
GC055	28.68 ± 0.21	28.35 ± 0.36	20.91 ± 1.37	0.24 ± 0.04	2.26 ± 0.14	8.10 ± 0.39	**59.86**
GC056	33.19 ± 1.72	21.26 ± 0.78	11.28 ± 0.15	0.22 ± 0.01	1.77 ± 0.34	6.55 ± 1.13	**41.08**
GC058	28.95 ± 1.13	20.32 ± 0.41	12.00 ± 0.71	0.45 ± 0.03	1.98 ± 0.62	8.00 ± 0.93	**42.75**
GC060	34.85 ± 1.29	47.34 ± 4.35	77.58 ± 4.11	2.41 ± 1.48	6.59 ± 0.40	11.68 ± 1.46	**145.60**
GC061	43.83 ± 1.06	43.48 ± 2.39	18.81 ± 4.60	1.82 ± 0.26	2.91 ± 0.43	11.95 ± 0.55	**78.97**
GT001	37.84 ± 1.55	48.18 ± 2.22	70.98 ± 6.19	0.81 ± 0.01	4.29 ± 0.26	9.37 ± 0.36	**133.63**
GJ002	28.79 ± 0.67	35.76 ± 2.99	83.07 ± 8.64	ND	5.93 ± 0.56	6.79 ± 0.44	**131.55**
GJ003	32.52 ± 0.80	36.95 ± 0.88	94.72 ± 1.33	ND	6.54 ± 0.12	6.43 ± 0.63	**144.64**
GJ013	32.52 ± 0.80	40.05 ± 3.54	9.91 ± 0.20	2.25 ± 0.05	7.27 ± 0.47	7.44 ± 2.16	**66.92**

[a] Each of the tea leaves (1 g) was extracted by 100 mL of 75% ethanol at 60 °C for 30 min. Each value represents the mean ± SE of five individual determinations. ND, not detectable.

ethanol. It was ~4.7-fold higher than that extracted by boiling water. There were more catechins and caffeine in the tea sample extracted by 65−85% ethanol than in that extracted by boiling water (ranging from 1.53- to 10.52-fold). Therefore, we continued to use boiling water and 75% ethanol to extract samples and analysis by HPLC.

Determination of Caffeine and Catechins in Commercial Tea Samples. Thirty-one commercial tea products were analyzed by the HPLC method as described above. The levels of their caffeine and catechins in water and 75% ethanol extracts were estimated and are summarized in **Tables 2** and **3**, respectively. Many biological effects of green tea appear to be mediated by its major polyphenolic constituents, EGCG and EGC. **Table 2** shows the polyphenol composition in water extract of various teas. The level of EGCG in black tea and pu-erh tea extracted by boiling water was very low and similar to the level of other catechins. All green tea (excluding GC048 and GC056) samples contain more EGCG than oolong, black, and pu-erh tea samples. This study also found that the noncultivated (wild growth) Lu-Shan cloud tea (GC060) contained more EGCG, EGC, ECG, and total catechins than other commercial teas including the cultivated Lu-Shan cloud tea (GC061). This situation is similar to the fresh tea leaves analyzed by HPLC; the levels of EGCG and total catechins in wild tea (also wild growth) were higher than in other fresh tea leaves (TTES 8 and TTES 12) (data not shown). **Table 3** shows the polyphenol composition of various teas in 75% ethanol extract. The polyphenols (including caffeine and five catechins) in tea samples extracted by 75% ethanol were higher than in that extracted by boiling water. The level of EGCG in green tea manufactured from old tea leaves (GC060, GC061, GT001, GJ002, GJ003, and GJ013) was higher than in other tea samples. In addition, Taiwan oolong (OT013, OT014, OT015, and OT016) extracted by 75% ethanol has a higher level of EGC than all the other tea samples (**Table 3**). Three kinds (TTES 8, TTES 12, and wild tea) of tea manufactured by different fermentation processes were analyzed by HPLC. EGCG and total catechins were very low in fresh tea leaves but higher in unfermented green tea than in the others. The level or degree of fermentation was inverse to the levels of EGCG and total catechins. In addition, the levels of EGCG and total catechins were in the order green tea > oolong tea > fresh leaves > black tea (data not shown); these results were similar to those observed in commercial teas.

Effects of Cultivating Location on the Catechins in Different Commercial Teas. Total catechins in teas from different location extracted by boiling water and 75% ethanol are illustrated in parts A and B, respectively, of **Figure 3**. The old leaf green tea produced from Lu Mountain in China, Shan-Xia in Taiwan, and Japan decoct tea had highest EGCG and total catechins. The young leaf green tea from Zhe-Jinag, Huang Mountain, and E-Mei Mountain contained lower EGCG and total catechins than other green teas, Wuu-Yi rock tea 1 (high quality), and Taiwan oolong tea. The Taiwan oolong tea extracted by 75% ethanol contained more EGC, and the levels of total catechin were similar to those of the green tea from Lu Mountain, Shan-Xia, and Japan decoct tea. The levels of catechins in oolong tea were in the order Taiwan oolong tea > Wuu-Yi rock tea 1 (high quality) > Wuu-Yi rock tea 2 (low quality). The average of total catechins in five high-quality rock

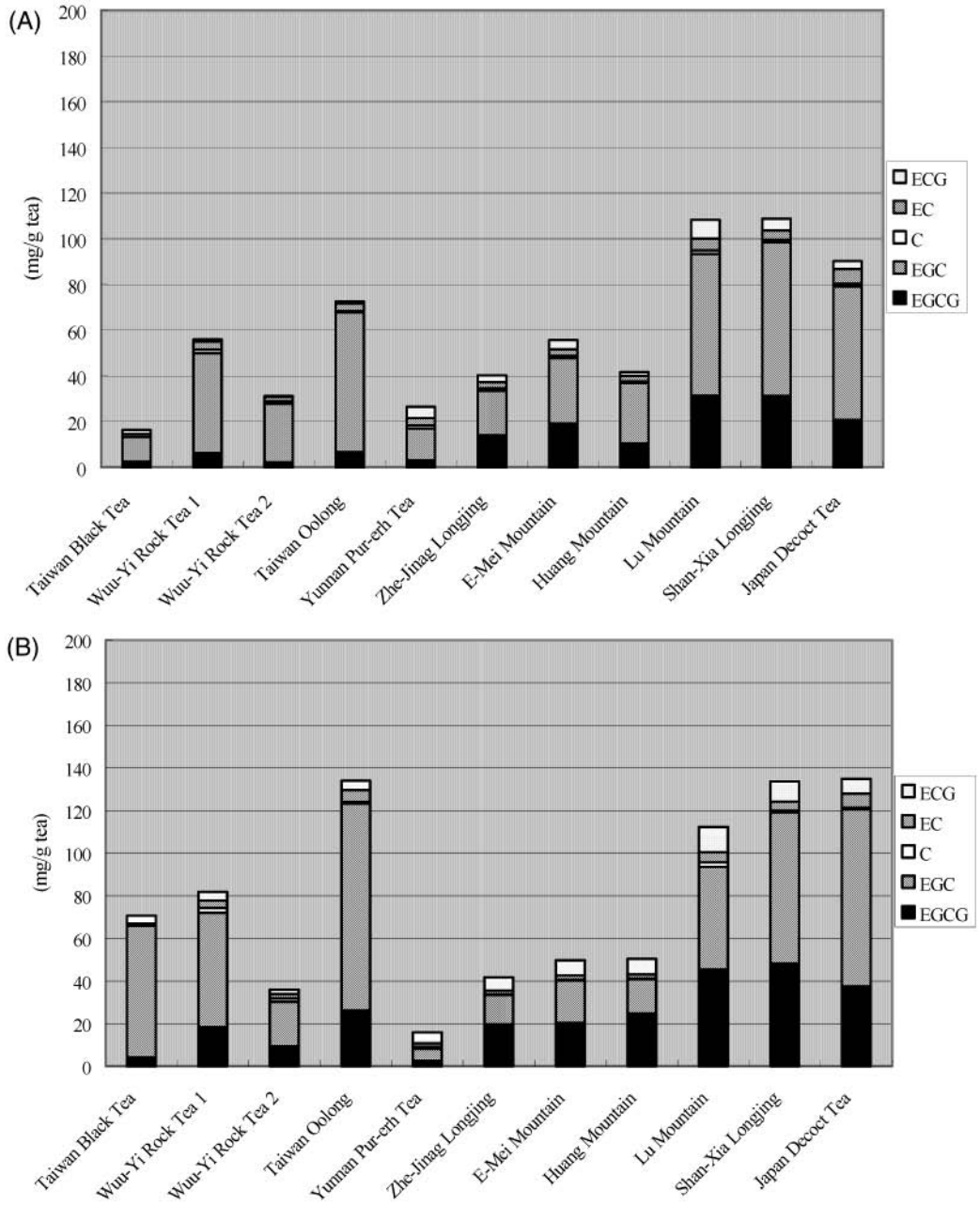

Figure 3. Amounts (mg/g) and proportions of different catechins in commercial teas from different locations: (A) teas were extracted by boiling water for 30 min; (B) teas were extracted by 75% ethanol at 60 °C for 30 min. Wuu-Yi rock tea 1 was a high-quality oolong tea as labeled in the local market, and Wuu-Yi rock tea 2 was of low quality. Taiwan black tea is BT001. Wuu-Yi rock tea 1 includes OC011, OC012, OC013, OC014, and OC032. Wuu-Yi rock tea 2 includes OC015, OC016, OC017, and OC018. Taiwan oolong tea includes OT013, OT014, OT015, and OT016. Yunnan pu-erh tea includes PC016, PC023, and PC024. Zhe-Jinag longjing includes GC048 and GC058. E-Mei mountain includes GC027, GC028, and GC029. Huang mountain includes GC055 and GC056. Lu mountain includes GC060 and GC061. Shan-Xai longjing is GT001. Japan decoct tea includes GJ002, GJ003, and GJ013.

teas was 1.80−2.27-fold higher than in four low-quality rock teas. Yunnan pu-erh tea contained the lowest levels of catechins, and the Taiwan black tea contained lower levels of catechins in water extract but higher levels in 75% exthanol extract than the green tea from Zhe-Jinag, Huang Mountain, E-Mei Mountain, and Wuu-Yi rock tea (low quality).

Effects of Cultivating Age on Tea Polyphenols and Caffeine in Commercial and Fresh Teas. Table 2 shows that green tea manufactured from young tea leaves (GC027, GC028, GC029, GC048, GC053, GC055, GC056, and GC058) contained less EGCG and total catechins than tea manufactured from old leaves (GC060, GC061, GT001, GJ002, GJ003, and GJ013). The levels of caffeine and catechins in seven fresh leaves were estimated and are summarized in **Table 4**. The level of caffeine in all old leaves (fifth to seventh leaves) was less than that in young leaves (apical bud and the two youngest leaves) as shown in **Table 4**. In contrast, the levels of EGCG, EGC, and EC in all old leaves were higher than in young leaves. The level of C in old leaves was higher than in young leaves (excluding TTES 12). **Table 4** shows the level of ECG in fresh tea leaves; the young leaves are higher than old leaves for TTES 5 and TTES 12, but the young leaves are lower than old leaves for TTES 1, TTES 4, and Wuu-Yi. The level of total catechins in fresh leaves extracted by boiling water is illustrated in **Figure 4**. It is interesting to see the effects of cultivating age (plucking position) on the caffeine and catechins in tea leaves. We further

Table 4. Levels of Caffeine and Catechins in Fresh Tea Leaves

tea sample[a,b]	caffeine		EGCG		EGC		C		EC		ECG	
	young	old	young	old	young	old	young	old	young	old	young	old
TTES 1	17.81 ± 0.87	13.04 ± 1.30[d]	1.27 ± 0.17	2.35 ± 0.09[d]	6.73 ± 0.40	9.64 ± 0.86[d]	ND[c]	ND	ND	0.62 ± 0.01[d]	ND	ND
TTES 2	25.98 ± 4.75	15.23 ± 1.58	1.41 ± 0.39	1.50 ± 0.45	12.95 ± 8.83	21.55 ± 10.5	ND	0.21 ± 0.09[d]	0.63 ± 0.14	2.90 ± 0.75[d]	0.90 ± 0.51	ND[d]
TTES 3	16.24 ± 2.91	15.31 ± 1.33	0.97 ± 0.66	2.84 ± 1.00[d]	ND	9.61 ± 1.80[d]	ND	0.35 ± 0.26[d]	0.63 ± 0.34	1.14 ± 0.23[d]	0.57 ± 0.30	0.58 ± 0.30
TTES 4	14.53 ± 2.68	11.88 ± 0.40	0.25 ± 0.28	0.87 ± 0.21	9.98 ± 1.12	11.83 ± 0.68[d]	ND	ND	ND	0.95 ± 0.19[d]	ND	ND
TTES 5	16.64 ± 1.90	11.43 ± 1.96[d]	1.10 ± 0.45	1.21 ± 0.54	ND	12.87 ± 1.41[d]	ND	0.10 ± 0.05[d]	0.93 ± 0.21	1.52 ± 0.15[d]	0.39 ± 0.18	0.27 ± 0.01
TTES 12	27.38 ± 0.63	14.85 ± 0.28[d]	3.51 ± 1.00	5.00 ± 0.53[d]	11.22 ± 0.30	20.96 ± 0.68[d]	1.15 ± 0.07	0.05 ± 0.01[d]	0.54 ± 0.05	1.48 ± 0.06[d]	1.33 ± 0.13	0.76 ± 0.02[d]
Wuu-Yi	18.17 ± 1.19	17.12 ± 1.26[d]	1.18 ± 0.99	8.10 ± 1.28[d]	5.59 ± 2.55	42.19 ± 5.86[d]	0.48 ± 0.23	0.73 ± 0.04	1.60 ± 1.65	3.76 ± 0.37[d]	1.09 ± 0.31	2.46 ± 0.30[d]

[a] Levels of caffeine and catechins were determined by HPLC method as described under Materials and Methods. Each tea sample (1 g) was extracted by 100 mL of boiling water for 30 min. Each value represents the mean ± SE of five individual determinations. [b] Each value was expressed as mg/g of tea leaf. The species of the tea samples were obtained by a series of breedings performed by the Taiwan Tea Experiment Station (TTES), Tao-Yuan, Taiwan. [c] ND, not detectable. [d] Compared with young leaves, $p < 0.05$ (Student's t test).

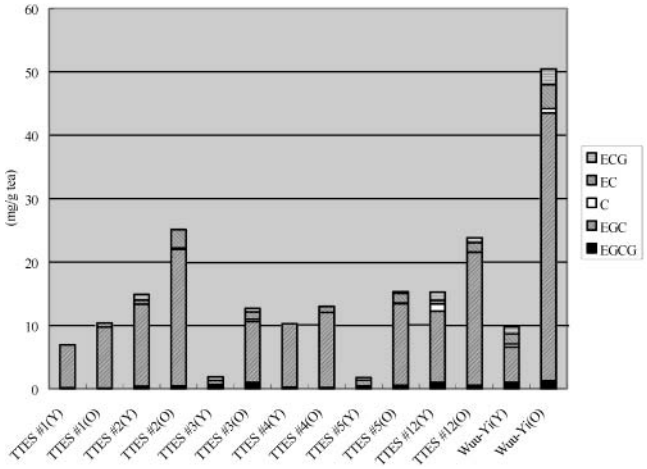

Figure 4. Total catechins in different fresh leaves. The amounts (mg/g) and proportions of different catechins in different fresh tea leaf species are plotted. The fresh leaves were extracted by boiling water for 30 min.

plucked fresh leaves from the same tea tree (TTES 12) but from a different plucking position of leaves. **Figure 5** shows the caffeine and catechins in different plucking positions of leaves (from the top of the bud to the oldest leaves were designated the 1st−15th leaves). Caffeine in the 1st−3rd leaves was higher than in the 4th−15th leaves. EGCG, EGC, ECG, and EC in three youngest leaves (1st−3th) were very low, and the 9th leaves had the highest level. Levels of C in the samples (TTES 12) were very low (almost not detected) and higher in the 2nd and 6th leaves.

Hydroxyl Radical Scavenging Effects of Tea Extracts. The antioxidant activity of six commercial tea extracts (extracted by boiling water and 75% ethanol) were assayed by Fenton reaction system as described under Materials and Methods. The effects of tea extracts on hydroxyl radical-induced DNA damage were investigated using $H_2O_2/FeSO_4$ in this study. Treatment of pcDNA-3 plasmid with H_2O_2 and $FeSO_4$ concentration dependently relaxed the supercoiled form DNA (data not shown). However, cotreatment with tea crude extracts exerted a protective effect from the $H_2O_2/FeSO_4$-induced DNA damage in a concentration-dependent manner (**Figure 6**). The arbitrary values coming from densitometric analysis represent the supercoiled forms plasmid DNA to relaxed forms plasmid DNA, and the relative level calculated as the ratio of supercoiled/relaxed observed in the control group is set at 1. The results in **Figure 6C** show all of the pu-erh tea crude extracts protected the plasmid DNA damage as well as the control at a concentration of 100 μg/mL. The antioxidative activity of tea extract was in the order pu-erh tea > green tea > black tea > oolong tea.

Suppressing Effects of Tea Extracts on LPS-Induced NO Production in Macrophages. The results of treatment with LPS only or along with the tested crude extract are shown in **Figure 7**. All crude extracts reduced NO generation at a concentration of 50 μg/mL. The pu-erh and black tea water extracts had stronger activities than green and oolong tea extracts to inhibit LPS-induced NO generation. Samples extracted by 75% ethanol increased the activities of suppression, especially green tea. The nitric oxide suppressing effect of tea extracts was in the order pu-erh tea ≥ black tea > green tea > oolong tea.

Induction of Apoptosis by Tea Extracts. Physiological cell death was characterized by apoptotic morphology, including chromatin condensation, membrane blebbing, internucleosomal degradation of DNA, and apoptotic body formation. In each case, nucleosomal DNA ladders, which are typical of apoptosis, were visible on agarose gel after staining with ethidium bromide. After treatment of HL-60 cells with 50 μg/mL various tea extracts or 0.05% DMSO (as control) for 24 h, the genomic DNA from cells was subjected to agarose gel electrophoresis. A clear DNA fragmentation ladder was found in agarose gels (**Figure 8**). In contrast, the intact genomic DNA was found in control. Oolong and green tea extracts (50 μg/mL) expressed a very strong effect to digest genomic DNA, which was evident at 24 h. The black tea ethanol extracts expressed a weak effect to induce a DNA fragmentation ladder (**Figure 8**).

To investigate the induction of the sub-G1 cell population, the DNA content of HL-60 cells treated with various tea extracts at 12.5, 25, and 50 μg/mL concentrations was analyzed by flow cytometry. The sub-G1 (sub-2N) DNA peak, which has been suggested to be the apoptotic DNA (*31*), was detected in cells that were stained with propidium iodide. As shown in **Table 5**, the percentages of apoptotic HL-60 cells were dose-dependently induced by tea extracts. The ability of tea extract to induce HL-60 cell apoptosis was in the order green tea > oolong > black tea > pu-erh tea (**Table 5**). The green and oolong tea extracts extracted by 75% ethanol were more effective than that extracted by boiling water.

DISCUSSION

Catechins are associated with the bitter and astringent taste of green tea. Tea has been considered to be a crude medicine in China for more than 4000 years. Various pharmacological effects such as protection of blood vessels, reduction of serum cholesterol levels, and prevention of arteriosclerosis were reported as integrated effects (*32*). Their action mechanisms of anticarcinogenesis have been attributed to the competitive inhibition of cytochrome P450 involved in the bioactivation of various carcinogens and the antioxidant properties of the scavenger of reactive oxygen species (*6*). The major active principles of tea have been identified as caffeine and catechins.

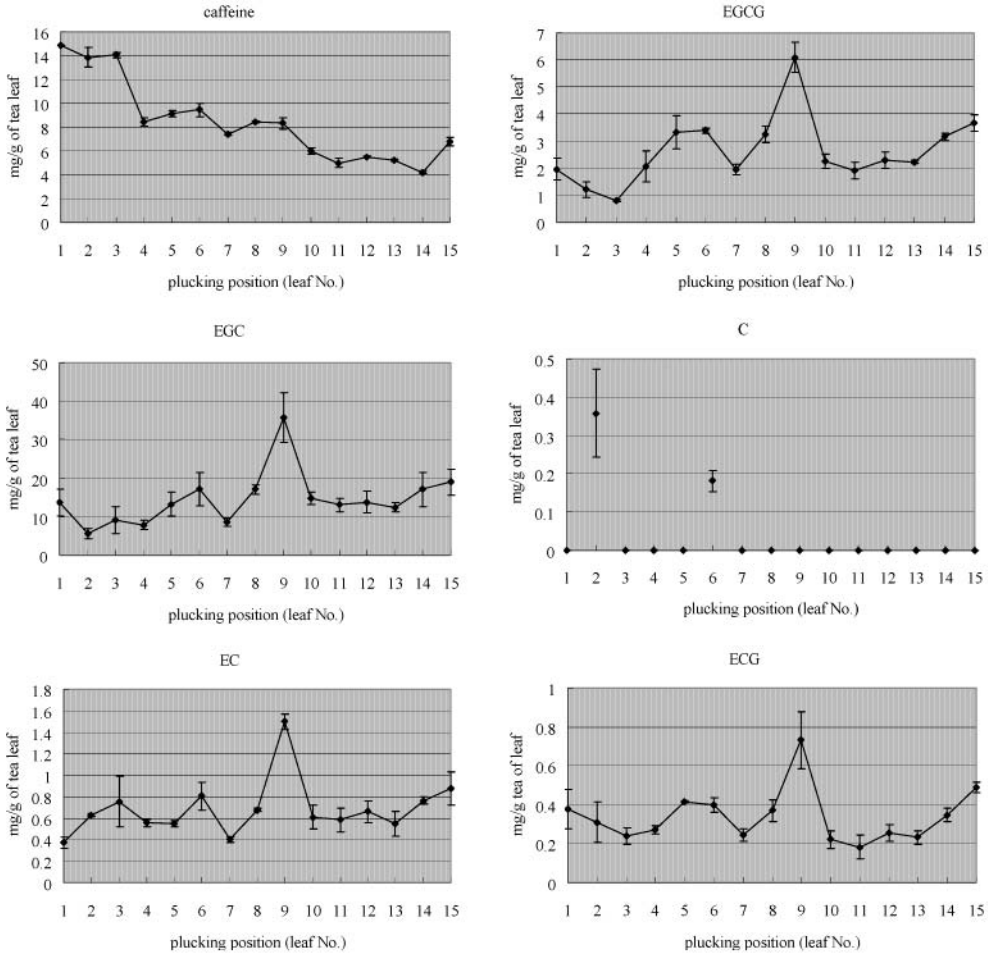

Figure 5. Caffeine and catechins spread in different leaves from different plucking positions. Each value represents the mean ± SE of three individual determinations.

In the present study, we used an isocratic HPLC procedure for the simultaneous determination of caffeine and catechins in tea and fresh tea leaf samples. The applicability and reproducibility of this method have been evaluated and assessed by the estimations of 31 commercial tea products (**Tables 2** and **3**). The analytical data seem to be reliable and reproducible with five repetitions. We found that the highest levels of EGCG and catechins can be detected when the sample was extracted by 75% ethanol (**Figure 2**). It could be suggested that the polarities of catechin are closer to those of ethanol, but the sample extracted by >85% ethanol could not detect more EGCG and catechins; this might be due to the lack of sufficient water to assist tea leaves in spreading the matrix structures.

It appears that the level of catechins in commercial tea is in the order green tea > oolong tea > black tea. The green tea polyphenol can be oxidized by polyphenol oxidase, and further polymerizations lead to theaflavins, thearubigins, and compounds of higher molecular mass when manufactured by fermentation (*1*, *33*, *34*). Our study systematically analyzed the samples from the same tea plant but manufactured by different degrees of fermentation. The results are similar to those of previous studies showing that the level of EGCG and catechins is in the order green tea > oolong tea > black tea, but the fresh tea leaves contain much lower EGCG and catechins than green and oolong teas (data not shown). It could be suggested that the manufacturing process of the fresh leaves to green tea might increase the level of catechins released from tea leaves when they were extracted.

Our studies demonstrated that green tea could be classified into two groups including young leaf green tea (Zhe-Jiang Longjing, E-Mei Mountain, and Huang Mountain) and old leaf green tea (Lu Mountain, Shan-Xia Longjing, and Japan decoct tea). The young leaf green tea contains lower levels of EGCG and total catechins than old leaf green tea and some oolong teas (**Figure 3**). The results are similar to that in fresh tea leaves when analyzed by HPLC. Seven fresh young and old leaves were analyzed, and the level of EGCG and total catechins is in the order old leaves > young leaves (**Figure 4**). For the first time, our study analyzed different plucking positions of leaves from the same tea tree (TTES 12). Then, we could know the change of catechins and caffeine in tea plants (**Figure 5**). It appeared that the formation of caffeine showed a peak in the first leaves; the production of the major catechins such as EGCG, EGC, ECG, and EC was greatest at the ninth leaves.

To compare the biological functions of different commercial tea extracts, the pu-erh and black teas have shown strong effects on the hydroxyl radical scavenging and nitric oxide suppression (**Figures 6** and **7**), and the green tea and oolong tea have strong effects on the induction of apoptosis (**Figure 8** and **Table 5**). In previous studies, the theaflavins in black tea and catechins in green tea were equally effective antioxidants (*35*). The pu-erh tea, unlike black tea (containing theaflavins) and green tea (containing catechins), has strong effects on antioxidation and inhibition of LPS-induced NO generation. It seems that some compounds other than catechins in pu-erh tea but not in green or black tea are strong NO-suppressing agents. The chemical

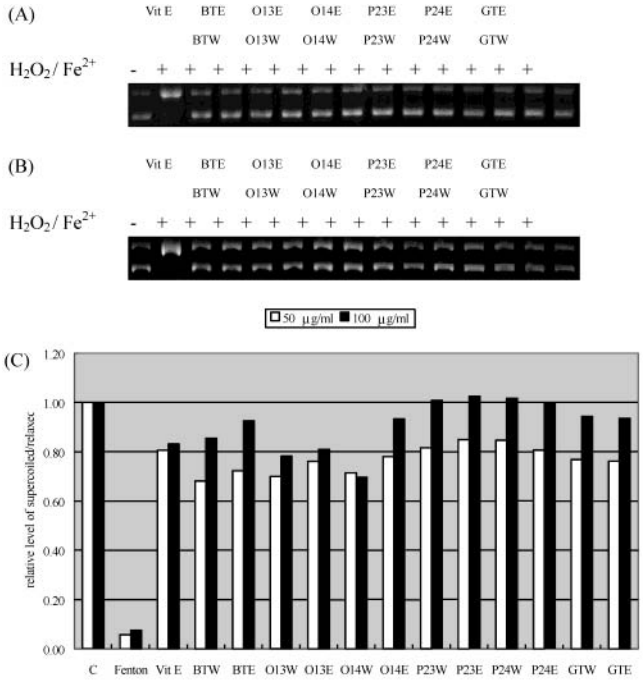

Figure 6. Hydroxyl radical scavenging effects of teas: (A) Protection of plasmid DNA damage by different tea extracts (100 μg/mL); (B) protection of plasmid DNA damage by different tea extracts (50 μg/mL); (C) relative level calculated as the ratio of supercoiled to relaxed forms coming from densitometric analysis. The assay system was described under Materials and Methods. The ratio of supercoiled/relaxed observed in control group is set at 1.00. Abbreviations: C, blank control system; Fenton, Fenton reaction mixture containing plasmid DNA as positive control; Vit E, vitamin E; BTW, black tea water, extract; BTE, black tea, 75% ethanol extract; O13W and O14W, oolong tea, water extracts; O13E and O14E, oolong tea 75% ethanol extracts; P23W and P24W, pu-erh tea, water extract; P23E and P24E, pu-erh tea, 75% ethanol extract; GTW, green tea, water extract; GTE, green tea, 75% ethanol extract.

Table 5. Percentage of Sub-G1 Cells Dose-Dependently Treated with Tea Crude Extracts and Analyzed by Flow Cytometry: Control, 2.77%; 0.5% DMSO, 2.97%[a]

	concn of tea extracts					
	water extract			75% ethanol extract		
sample	12.5 g/mL	25 g/mL	50 g/mL	12.5 g/mL	25 g/mL	50 g/mL
BT001	2.46	4.26	13.07	2.58	5.01	18.44
OT013	3.94	6.63	25.17	5.65	11.96	30.58
OT014	4.21	10.85	41.51	3.71	10.27	51.52
PT023	3.78	5.91	5.05	3.57	3.59	5.43
PT024	2.93	3.42	12.29	4.23	4.28	9.60
GT001	4.47	12.37	55.46	6.06	20.22	66.90

[a] Determination of sub-G1 cells in control and tea crude extract treated HL-60 cells by flow cytometry. BT001, black tea; OT013 and OT014, oolong tea; PC023 and PC024, pu-erh tea; GT001, green tea.

nature of polymerized tea catechins in pu-erh tea is not well-characterized. The reactive molecules that are responsible for NO suppression are unknown. These polymerized tea catechins may act through directly scavenging the generated NO or indirectly inhibiting the NO-generating enzyme inducible nitric oxide synthase system. This interesting problem is worthy of further study. The activities of tea on the induction of apoptosis are in the order green tea > oolong tea > black tea > pu-erh tea, and the results correspond to the level of EGCG and total

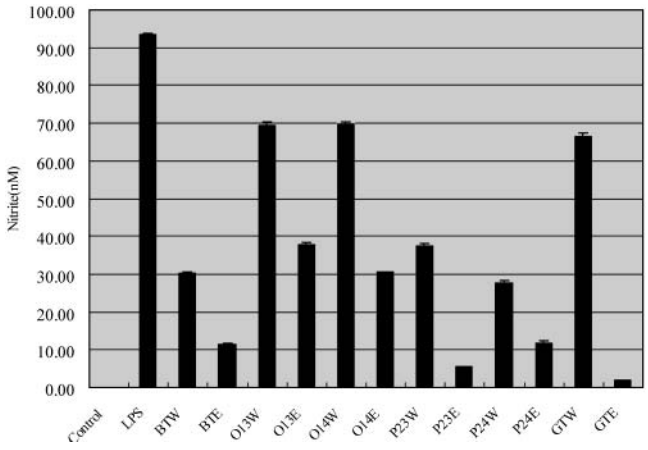

Figure 7. Suppressing effect of various tea extracts (50 μg/mL) on LPS-induced NO production in macrophages. Tea extracts (50 μg/mL) were cotreated with macrophages activated with LPS (50 ng/mL) for 16 h. The assay system was described under Materials and Methods. Abbreviations: C, blank control system; LPS, cells treated with LPS only; BTW, black tea water, extract; BTE, black tea, 75% ethanol extract; O13W and O14W, oolong tea, water extracts; O13E and O14E, oolong tea 75% ethanol extracts; P23W and P24W, pu-erh tea, water extract; P23E and P24E, pu-erh tea, 75% ethanol extract; GTW, green tea, water extract; GTE, green tea, 75% ethanol extract.

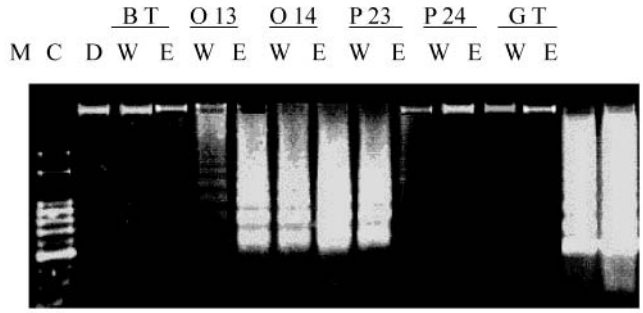

Figure 8. Induction of the DNA fragmentation ladder by tea extracts (50 μg/mL) in HL-60 cells. The assay system was described under Materials and Methods. Abbreviations: C, blank control system; D, treated 0.05% DMSO only as control; BT, black tea (BT001); O13, oolong tea (OT013); O14, oolong tea (OT014); P23, pu-erh tea (PC023); P24, pu-erh tea (PC024); GT, green tea (GT001); W, water extract; E, 75% ethanol extract.

catechins in teas. However, in our previous studies, the activities of theaflavins in black tea and theasinesin A in oolong tea are more effective in the induction of cell apoptosis than are catechins in green tea (*28*). Therefore, there could be some unknown compounds in tea playing a part in the induction of apoptosis.

The biological functions of pu-erh tea extracts are interesting; they were quite active in suppressing LPS-induced NO generation (**Figure 7**) but completely inactive in inducing apoptosis (**Figure 8**). It appears that pu-erh tea extract contains very little or no monomeric catechins. Therefore, the polymerized catechins may be responsible for these two biological functions. The polymerized catechins may not be permeable to the cell membrane and cannot get into the cell or mitochondria to affect the apoptotic processes. On the other hand, the polymerized catechins may block LPS to bind its receptor on the cell surface, which leads to suppression of the signal transduction pathways for inducible nitric oxide synthase (iNOS) gene transcription.

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