JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

HPLC Analysis of Naturally Occurring Methylated Catechins, 3′′- and 4′′-Methyl-epigallocatechin Gallate, in Various Fresh Tea Leaves and Commercial Teas and Their Potent Inhibitory Effects on Inducible Nitric Oxide Synthase in Macrophages

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(−)-Epigallocatechin-3-gallate (EGCG), a major polyphenol of green tea, undergoes substantial biotransformation to species that includes the methylated compounds. Recent studies have demonstrated that the methylated EGCG has many biological activities. In this study, we have investigated the composition of the three O-methylated EGCG derivatives, (−)-epigallocatechin-3-*O*-(3-*O*-methyl)gallate (3′′-Me-EGCG), (−)-epigallocatechin-3-*O*-(4-*O*-methyl)gallate (4′′-Me-EGCG) and (−)-4′-methyl epigallocatechin-3-*O*-(4-*O*-methyl)gallate (4′,4′′-di-Me-EGCG) in tea leaves which were picked from various species and at various seasons, ages of leaves, locations, and fermentation levels. Higher levels of 3′′-Me-EGCG and 4′′-Me-EGCG were detected in Chinshin-Kanzai (a species of *Camellia sinensis*) cultivated in the mountain area of Sansia, Taipei County, Taiwan. Also, these O-methylated EGCG levels were found to be higher in autumn and winter than in spring and summer. The young leaves were found to be richer in the O-methylated compounds than old leaves and the amount of O-methylated EGCG was higher in unfermented longjin green tea than in semifermented oolong tea. However, the fermented black tea and puerh tea did not contain these compounds. 4′,4′′-diMe-EGCG could not be detected in either fresh tea leaves or commercial tea leaves. We also found that 3′′-Me-EGCG has a higher inhibitory effect on the nitric oxide generation and inducible nitric oxide synthase (iNOS) expression as compared with EGCG, while 4′′-Me-EGCG and 4′,4′′-di-Me-EGCG were less effective.

KEYWORDS: EGCG; methylation; 3′′-Me-EGCG; 4′′-Me-EGCG; Chinshin oolong; Chinshin-Kanzai; iNOS

INTRODUCTION

Tea (*Camellia sinensis*) is one of the most popular beverages in the world because of its attractive aroma, taste, and healthy effects. Hundreds of teas are now produced; generally, they are classified into three major categories: nonfermented green tea; partially fermented oolong tea; and the fully fermented black or puerh tea. Tea contains various beneficial constituents, and several in vitro and in vivo studies have demonstrated that constituents of tea exhibit biological and pharmacological properties. These constituents have been reported to have antioxidative activity (*1*−*5*), antimutagenic effects (*6*−*9*), anticarcinogenic effects (*6*, *10*−*13*), and antiallergic effects (*14*−*18*). The polyphenols are the most significant group of tea components, especially certain catechins which play a significant role in these actions. The major catechins in tea leaves are (−)-epigallocatechin 3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin 3-gallate (ECG), (−)-epicatechin (EC), (−)-gallocatechin (GC), and (+)-catechin (C).

Metabolism of tea catechins results in extensive methylation and glucuronidation (*19*, *20*). These reactions may play key roles in determining the specific biological and pharmacological activities of catechins. Recent research has shown that the O-methylated derivatives of EGCG, 3′′-Me-EGCG and 4′′-Me-EGCG, inhibit type I and type IV allergies more effectively than EGCG (*21*, *22*). However, the understanding of the biological activities of methylated EGCG is still very limited.

Nitric oxide (NO) plays diverse roles in physiological and pathological processes. During immune and inflammatory response, for example in asthma, NO is generated at relatively high and sustained levels by the inducible form of nitric oxide synthase (iNOS) (*23*−*25*). The high concentrations of NO in asthma, which are believed to reflect upregulation of inducible NO (iNO) by proinflammatory cytokines, may produce various deleterious effects. These include increased vascular permeability, damage to the airway epithelium, and promotion of inflammatory cell infiltration (*23*, *24*). Therefore, inhibition of iNO may be a useful therapeutic strategy in asthma.

In the present study, we investigated whether O-methylated EGCG exists in the tea tree, and separated the catechins,

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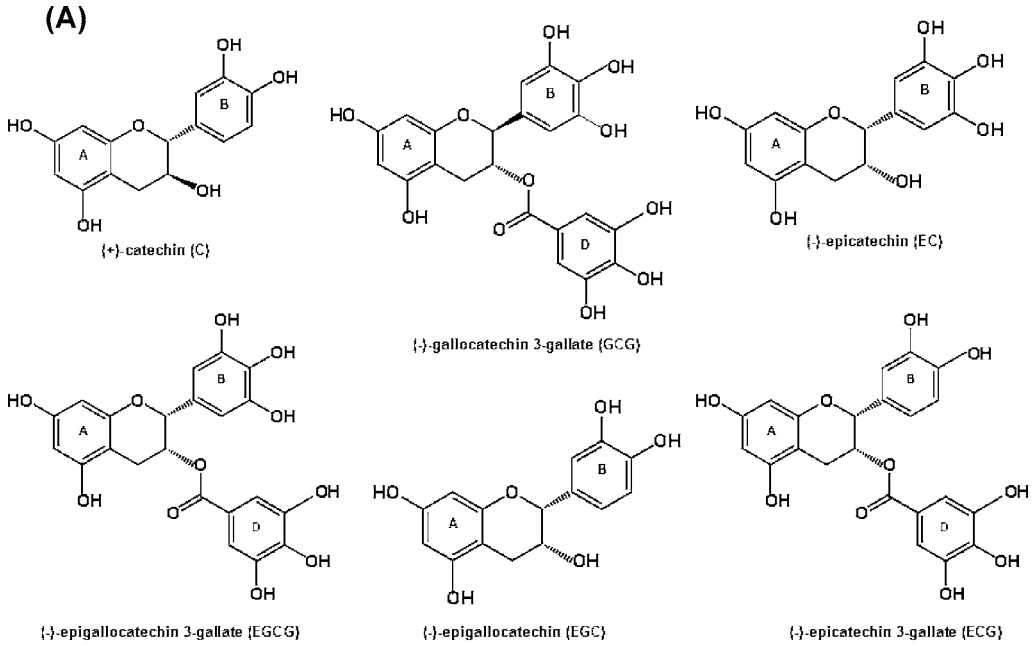
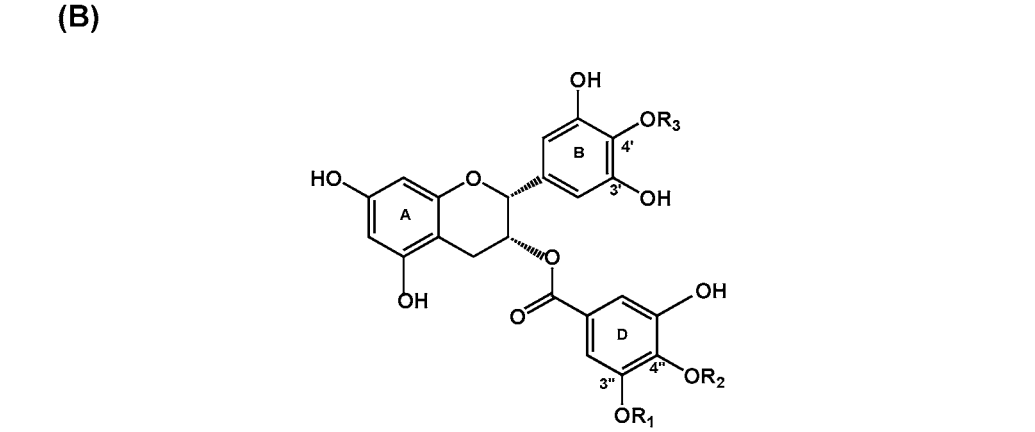
10.1021/jf0507442 CCC: $30.25
Published on Web 08/12/2005





	R_1	R_2	R_3
EGCG	H	H	H
3″-Me-EGCG	CH_3	H	H
4″-Me-EGCG	H	CH_3	H
4′,4″-diMe-EGCG	H	CH_3	CH_3

Figure 1. Chemical Structures of catechins. (A) Tea catechins. (B) EGCG and O-methylated derivatives of EGCG.

especially certain O-methylated EGCG derivatives, from various parts, locations, and seasons of the fresh tea leaves, and in commercial tea, by HPLC analysis. We also studied the inhibition of NO production by O-methylated EGCG in LPS-activated macrophage. These results can provide beneficial information in the prevention of allergic disorders.

MATERIALS AND METHODS

Chemicals and Reagents. (−)-Epigallocatechin 3-gallate (EGCG), (−)-epigallocatechin (EGC), (+)-catechin (C), (−)-epicatechin (EC), (−)-epicatechin 3-gallate (ECG), (−)-gallocatechin 3-gallate (GCG) (**Figure 1A**) and lipopolysaccharide (LPS, *Escherichia coli* 0127:E8), were purchased from Sigma Chemical Co. (St. Louis, MO). (−)-Epigallocatechin-3-*O*-(3-*O*-methyl)gallate (3″-Me-EGCG), (−)-epigallocatechin-3-*O*-(4-*O*-methyl)gallate (4″-Me-EGCG), and (−)-4′-methyl epigallocatechin-3-*O*-(4-*O*-methyl)gallate (4′,4″-diMe-EGCG) were kindly supplied by Professor M. Sano. Sodium dihydrogen phosphate, ethylenediaminetetraacetic acid, disodium salt, and acetonitrile were purchased from E. Merck Co. (Darmstadt, Germany).

Tea and Tea Leaf Samples. In this study, fresh Chinshin-Kanzai tea leaves were from Sansia township and the Sinpu substation of the Taiwan Tea Experiment Station (TTES); fresh Chinshin oolong tea leaves were from different townships (Tongting, Pinglin, and Mingjian) located in Taiwan. The other 16 species of fresh tea leaves were plucked from the Pinglin substation of the TTES. The young leaves contain the apical bud and the two youngest leaves, and the old leaves are from the tenth to the fifth leaf of different varieties. The collected tea leaves were dried at 45 °C overnight in an electric oven with a rotating fan to keep the heat evenly distributed. The weight of the tea leaves was checked from time to time until a constant weight was reached.

Several commercial tea samples including lonjing, oolong, puerh, and black teas from different cities located in China, Japan, and Taiwan were analyzed. Oolong tea is a kind of commercial tea, while Chinshin oolong tea is the name of a tea plant species. These tea samples were collected in the local markets when the authors were traveling in these

cities. The chemical compositions of these tea samples varied with their species, cultivation conditions, such as weather, temperature, moisture, latitude, and season, and process of manufacturing. It has been established that the levels of polyphenols, especially catechins, are significantly affected by the production process.

Preparation of Extracts from Tea Leaves and Tea. Each of the dry tea leaves (0.5 g) was steeped in boiling distilled water (50 mL) for 3 min at 90 °C. The infusion was filtered with a 0.45 μm PVDF filter disk (Millipore, Bedford, MA). The filtrate was analyzed by HPLC as described below.

Reverse-Phase HPLC Analysis of Tea Polyphenols and O-Methylated EGCG. The determination of O-methylated EGCG was carried out by high-performance liquid chromatography (HPLC) with an electrochemical detector (ECD) as previously described (*26*). The HPLC used a 250 × 4.6 mm i.d., 5 μm Cosmosil 5 C18-MS packed column (Nacalei Tesque, Inc., Kyoto, Japan). Briefly, the mobile phase was 0.1 M sodium dihydrogen phosphate buffer (pH 2.5) containing 0.1 mM ethylenediamine tetraactic acid disodium salt (Na_2EDTA)/acetonitrile (87/13 v/v), and the flow rate was 1 mL/min. The column was maintained at 30 °C. The O-methylated EGCGs were detected electrochemically at an applied potential of 600 mV versus Ag/AgCl.

Cell Culture. RAW 264.7 derived from murine macrophages were obtained from the American Type Culture Collection (Rockville, MD). RAW 264.7 cells were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free heat-inactived fetal calf serum (Gibco), penicillin (100 unit/mL), and streptomycin (100 μg/mL).

Nitrite Assay. The nitrite concentration in the cultured RAW 264.7 cells medium was measured as an indicator of NO production, according to the Griess reaction. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance of the mixture at 550 nm was determined with a Dynatech MR-7000 enzyme-linked immunosorbant assay plate reader (Dynatech Labs, Chantilly, VA).

Western Blots. Total cellular extract was prepared using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4/150 mM NaCl/1% Triton X-100/1% deoxycholate/0.1% SDS/1% aprotinin). Total protein containing 50 μg of proteins was separated on 8% sodiumdodecyl sulfate polyacrylamide minigels and transferred to an Immobilon PVDF membrane (Millipore). The membrane was incubated overnight at 4 °C with 10% bovine serum albumin in phosphate-buffered saline to block nonspecific immunoglobulins and then incubated with anti-iNOS monoclonal antibody (Transduction Laboratories) and anti-α-tubulin monoclonal antibody (Oncogene Science). Inducible NO synthase and α-tubulin protein were detected by chemiluminescence (ECL, Amersham). Band intensities were quantified by densitometry (IS-1000 Digital Imaging System). The experiment was repeated three times with similar results.

RESULTS

HPLC Analysis of Authentic Standard Tea Polyphenols and O-Methylated EGCG. A mixture of authentic standard tea catechins including (−)-epigallocatechin (EGC), catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin 3-gallate (EGCG), (−)-gallocatechin 3-gallate (GCG), (−)-epigallocatechin-3-*O*-(4-*O*-methyl) gallate (4″-Me-EGCG), (−)-epigallocatechin-3-*O*-(3-*O*-methyl) gallate (3″-Me-EGCG), (−)-4′-methyl epigallocatechin-3-*O*-(4-*O*-methyl) gallate (4′,4″-diMe-EGCG), and (−)-epicatechin-gallate (ECG) was separated by isocratic HPLC combined with electrochemical detection (ECD) as described above, and a baseline resolution was achieved (**Figure 2A**). The structures of the O-methylated EGCG are shown in **Figure 1B**. The HPLC separation of these tea constituents (10 ng of each) was accomplished in 60 min.

Survey of Tea Polyphenols and O-Methylated EGCG in Fresh Tea Leaves of Taiwan Varieties. It has been reported that the Tongting oolong tea in Taiwan contains O-methylated EGCG derivatives (*21*, *26*). It is unclear whether the O-methylated EGCG is produced during the manufacturing processes or is naturally present in the tea leaves themselves. We analyzed the catechins and O-methylated EGCG of oolong fresh tea leaves from the different townships (Tongting, Pinglin, and Mingjian) located in Taiwan using HPLC. The levels of catechins and O-methylated EGCG in tea leaves are summarized in **Table 1**. This result demonstrates that the O-methylated EGCGs are present in the tea leaves themselves. We also analyzed the amounts of EGCG and O-methylated EGCG in different plucking positions (age of the leaves) and found that EGCG levels in the young leaves were much greater than in the older leaves, but the content of O-methylated EGCG in all kinds of tea showed regional diversity. However, for the Pinglin oolong tea leaves, the young leaves contain much more O-methylated EGCG compared with that in the older leaves.

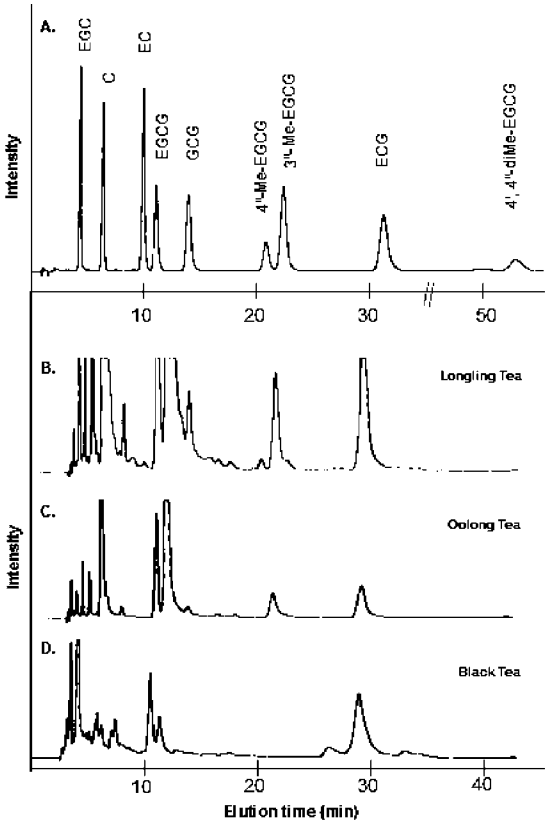


Figure 2. Isocratic HPLC separation of catechins and O-methylated EGCG in tea water extracts (TWE). The following sample solutions were analyzed: (A) mixture of authentic standard compounds, 10 ng of each; (B) oolong tea (Tongting, Nantou County, Taiwan), 0.01% TWE; (C) longjing green tea (Sansia, Taipei), 0.01% TWE; (D) black tea (Yunnan, China), 0.01% TWE.

In addition to the Chinshin oolong fresh tea leaves, we also examined the catechins and O-methylated EGCG of 16 different tea varieties from TTES (Pinglin, Taiwan). The levels of catechins and O-methylated EGCG are summarized in **Table 2**. The results indicate that each of the varieties has different levels of O-methylated EGCG, demonstrating that the O-methylated EGCG is present in the tea leaves naturally rather than produced during the manufacturing processes.

HPLC Separation of Catechins and O-Methylated EGCG in Chinshin Oolong Tea during Four Seasons from Mingjian Township, Taiwan. The composition of tea varies with species, season, age, climate, and horticultural practices. To this end, we analyzed the amount of O-methylated EGCG at various seasons. For commercial tea samples from Mingjian Township

Table 1. Catechin Levels of Various Ching-Hsin-Oolong Fresh Tea Leaves in Taiwan

		mg/g of tea leaf[a]								ratio of	
county		EGC	C	EC	EGC G	GCG	4″-Me-EGC G	3″-Me-EGC G	ECG	4″-MeE GCG/ total EGCG	3″-MeE GCG/ total EGCG
Tungting	old leaf	1.8 ± 0.2	0.1 ± 0.1	0.2 ± 0.0	1.8 ± 0.2	ND	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.13	0.04
	young leaf	2.8 ± 0.3	0.2 ± 0.1	0.7 ± 0.2	4.5 ± 1.4[b]	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.1[b]	1.0 ± 0.2	0.04	0.04
Pinglin	old leaf	2.9 ± 0.3	0.1 ± 0.1	0.3 ± 0.2	1.3 ± 0.0	ND	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.13	0.08
	young leaf	2.0 ± 0.3	ND	0.2 ± 0.0	3.0 ± 0.2[c]	ND	0.4 ± 0.0[c]	0.3 ± 0.1[c]	0.4 ± 0.0	0.11	0.04
Mingjian	old leaf	0.1 ± 0.0	ND	ND	0.2 ± 0.1	ND	0.1 ± 0.0	ND	ND	0.33	
	young leaf	0.2 ± 0.0	ND	0.1 ± 0.0	0.4 ± 0.1[b]	ND	0.1 ± 0.1	ND	0.2 ± 0.0	0.20	

[a] The values were presented as mean ± SE ($n = 3$); *n*, the number of determinations; ND, nondetectable. [b] $P \leq 0.05$ compared with old leaf. [c] $P \leq 0.01$ compared with old leaf.

Table 2. Catechin Levels of Fresh Tea Leaves in TTES

	mg/g of tea leaf[a]								ratio of	
TTES	EGC	C	EC	EGCG	GCG	4″-Me-EGCG	3″-Me-EGCG	ECG	4″-MeEGCG/ total EGCG	3″-MeEGCG/ total EGCG
1	0.7 ± 0.3	ND	0.1 ± 0.0	3.2 ± 0.3	ND	0.2 ± 0.0	0.2 ± 0.1	0.5 ± 0.0	0.06	0.05
2	1.6 ± 0.5	ND	0.3 ± 0.1	1.9 ± 0.2[b]	ND	0.1 ± 0.0[b]	0.2 ± 0.0	0.3 ± 0.0	0.03	0.09
3	1.2 ± 0.9	ND	0.2 ± 0.1	2.0 ± 0.2[b]	ND	0.1 ± 0.0[c]	0.2 ± 0.0	0.4 ± 0.0	0.06	0.07
4	0.5 ± 0.1	ND	0.1 ± 0.0	1.0 ± 0.1[b]	ND	0.1 ± 0.0[b]	0.1 ± 0.0[b]	0.2 ± 0.1	0.10	0.04
5	3.0 ± 0.6	0.1 ± 0.0	1.2 ± 0.1	1.0 ± 0.2[b]	ND	ND	0.2 ± 0.0	0.4 ± 0.1		0.15
6	0.7 ± 0.1	ND	0.1 ± 0.0	1.1 ± 0.2[b]	ND	ND	ND	0.3 ± 0.0		
8	4.6 ± 1.2	0.1 ± 0.1	0.4 ± 0.1	6.6 ± 0.6[b]	ND	ND	ND	0.5 ± 0.1		
9	1.3 ± 0.2	ND	0.1 ± 0.1	4.4 ± 3.9	ND	0.2 ± 0.0	0.2 ± 0.1	0.7 ± 0.1	0.05	0.04
10	4.4 ± 1.5	ND	0.5 ± 0.2	4.1 ± 0.5[c]	ND	0.3 ± 0.2	0.2 ± 0.0	0.5 ± 0.1	0.06	0.04
12	4.0 ± 0.3	ND	0.4 ± 0.1	2.3 ± 0.2[b]	ND	ND	0.1 ± 0.0	0.3 ± 0.0		0.05
13	2.6 ± 1.0	ND	0.3 ± 0.0	1.7 ± 0.2[b]	ND	ND	0.1 ± 0.0[c]	0.2 ± 0.0		0.05
14	3.8 ± 1.2	ND	0.6 ± 0.3	3.0 ± 0.6	ND	ND	0.1 ± 0.0	0.4 ± 0.1		0.03
15	4.3 ± 1.0	ND	0.4 ± 0.1	3.4 ± 0.3	ND	0.1 ± 0.1[c]	0.3 ± 0.0[c]	0.4 ± 0.0	0.03	0.07
16	4.6 ± 1.2	0.2 ± 0.1	0.4 ± 0.1	6.6 ± 0.6[b]	ND	ND	ND	0.5 ± 0.1		
17	1.3 ± 0.2	ND	0.1 ± 0.1	4.4 ± 4.0[c]	ND	0.2 ± 0.0	0.2 ± 0.5	0.7 ± 0.1	0.05	0.04
18	4.4 ± 1.5	ND	0.5 ± 0.2	4.1 ± 0.5	ND	0.3 ± 0.2	0.2 ± 0.0	0.5 ± 0.1	0.06	0.04

[a] The values were presented as mean ± SE ($n = 3$); *n*, the number of determinations; ND, nondetectable. [b] $P \leq 0.01$ compared with TTES1. [c] $P \leq 0.05$ compared with TTES1.

Table 3. Catechin Levels of Ching-Hsin-Oolong Tea in Various Seasons

	mg/g of tea[a]								ratio of	
	EGC	C	EC	EGCG	GCG	4″-Me-EGC G	3″-Me-EGC G	ECG	4″-MeEGCG/ total EGCG	3″-MeEGCG/ total EGCG
spring	4.2 ± 0.6	0.1 ± 0.0	1.0 ± 0.1	6.4 ± 1.3	0.1 ± 0.1	ND	0.2 ± 0.0	0.8 ± 0.3		0.03
summer	8.8 ± 1.1	0.1 ± 0.0	1.4 ± 0.1	6.9 ± 1.4	0.2 ± 0.0	ND	0.4 ± 0.0[b]	0.8 ± 0.1		0.05
fall	12.5 ± 1.5	0.4 ± 0.1	2.1 ± 0.5	10.6 ± 3.5	0.3 ± 0.2	0.3 ± 0.1[b]	0.4 ± 0.1[b]	1.4 ± 0.5	0.03	0.04
winter	12.1 ± 0.6	0.2 ± 0.0	2.0 ± 0.2	8.5 ± 1.5	0.2 ± 0.1	0.2 ± 0.0[b]	0.6 ± 0.1[b]	1.2 ± 0.2	0.02	0.06

[a] The values were presented as mean ± SE ($n = 3$); *n*, the number of determinations; ND, nondetectable. [b] $P \leq 0.01$ compared with spring.

in Taiwan, samples from four seasons of Chinshin oolong teas were analyzed by the HPLC method, and the levels of catechins and O-methylated EGCG are summarized in **Table 3**. The result shows the level of O-methylated EGCG is higher in autumn and winter than in spring and summer. For 3″-Me-EGCG especially, the level increases following the change of season.

HPLC Separation of Catechins and O-Methylated EGCG in Green Tea. Longjing tea and bi-luo-chun tea are classified as green tea in Taiwan and China, whereas the decocted tea is regarded as green tea in Japan. The representative HPLC patterns of Sansia longjing tea (Taiwan) are illustrated in **Figure 2B**. The levels of catechins and O-methylated EGCG are summarized in **Table 4**. The levels of O-methylated EGCG are highest in Sansia longjing tea (Taiwan) as compared with that in longjing tea (China), Bi-luo-chun green tea (Su-chou, China), and decocted tea (Japan). We also detected the amount of O-methylated EGCG in Sansia longjing fresh tea leaves, Chinshin-Kanzai (a species of *Camellia sinensis*), cultivated in the mountain area of Sansia, Taipei County, Taiwan. The results show that higher levels of 3″-Me-EGCG and 4″-Me-EGCG were detected in Chinshin-Kanzai. We found that the amount of 3″-Me-EGCG is higher than in Tongting oolong fresh tea leaves (**Table 1**).

HPLC Separation of Catechins and O-Methylated EGCG in Oolong Tea. Tongting oolong tea, high mountain tea, and Wuuyi oolong tea are classified as partially fermented teas in Taiwan and China. Oolong tea is the most popular tea consumed in Taiwan and southern China because of its attractive aroma and taste. The representative HPLC patterns of Tongting oolong tea are illustrated in **Figure 2C**, and the levels of catechins and O-methylated EGCG are summarized in **Table 5**. It seems that the levels of O-methylated EGCG are higher in Tongting oolong

Table 4. Catechin Levels of Green Tea in Taiwan, China, and Japan

	mg/g of tea[a]								ratio of	
	EGC	C	EC	EGC G	GCG	4″-Me-EG CG	3″-Me-EG CG	ECG	4″-MeE GCG/ total EGCG	3″-MeE GCG/ total EGCG
					Longjing Green Tea (Sansia, Taipei)					
Chinghsin-Kanzai										
old leaf	3.2 ± 0.2	ND	0.5 ± 0.1	2.3 ± 0.2	ND	ND	0.2 ± 0.0	0.4 ± 0.1		0.12
young leaf	1.7 ± 0.6	0.1 ± 0.0	0.5 ± 0.1	3.6 ± 0.9	0.1 ± 0.1	0.1 ± 0.0	0.5 ± 0.1[b]	1.2 ± 0.3	0.02	0.12
longjing tea	8.7 ± 1.5	0.5 ± 0.2	1.8 ± 0.4	12.6 ± 0.2	0.9 ± 0.1	0.4 ± 0.0	1.2 ± 0.2	2.3 ± 0.2	0.03	0.08
longjing tea powder	16.4 ± 0.5	0.2 ± 0.0	3.1 ± 0.3	15.3 ± 1.7[c]	0.7 ± 0.1	0.1 ± 0.0[d]	1.4 ± 0.1	2.2 ± 0.2	0.01	0.08
					Longjing Green Tea (Hsihu, China)					
longjing tea 1	0.6 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	6.6 ± 0.5[d]	0.5 ± 0.0	ND	ND	3.6 ± 0.3		
longjing tea 2	1.6 ± 0.2	0.2 ± 0.0	0.8 ± 0.0	6.5 ± 0.6[d]	0.7 ± 0.3	ND	0.1 ± 0.0[d]	2.2 ± 0.0		0.01
longjing tea 3	1.3 ± 0.3	0.2 ± 0.0	0.7 ± 0.1	6.8 ± 0.5[d]	1.1 ± 0.0	ND	0.1 ± 0.0[d]	2.2 ± 0.2		0.01
					Green Tea (China)					
Mengding mountain	1.4 ± 0.2	0.3 ± 0.0	0.9 ± 0.1	11.5 ± 0.3[d]	2.3 ± 0.4	ND	0.1 ± 0.0[d]	2.9 ± 0.1		0.01
Chu-Yeh-Ching	2.2 ± 0.4	0.3 ± 0.0	1.2 ± 0.2	12.6 ± 0.7	2.5 ± 0.5	ND	0.1 ± 0.0[d]	3.6 ± 0.3		0.01
Erh-Lang mountain	2.9 ± 0.1	0.3 ± 0.0	1.3 ± 0.1	13.1 ± 0.0[c]	1.3 ± 0.0	0.1 ± 0.0[d]	0.1 ± 0.1[d]	3.6 ± 0.1	0.01	0.01
Ching-Cheng tea 1	6.8 ± 0.2	0.4 ± 0.0	2.0 ± 0.1	12.1 ± 0.5	0.7 ± 0.1	0.4 ± 0.1	0.2 ± 0.1[c]	2.3 ± 0.2	0.03	0.02
Ching-Cheng tea 2	6.4 ± 0.2	0.5 ± 0.1	2.1 ± 0.2	11.9 ± 0.5	0.5 ± 0.0	0.2 ± 0.0[d]	0.1 ± 0.0[d]	2.4 ± 0.3	0.02	0.01
					Decocted Tea					
Osaka, Japan	9.9 ± 0.2	0.5 ± 0.0	2.9 ± 0.1	11.6 ± 0.8	0.5 ± 0.1	ND	0.1 ± 0.0[d]	2.4 ± 0.2	0.01	0.01
Shizuoka, Japan	8.8 ± 0.8	0.3 ± 0.1	2.3 ± 0.1	8.6 ± 1.0[d]	0.5 ± 0.1	ND	0.1 ± 0.0[d]	1.6 ± 0.2		0.01
Shizuoka, Japan	5.3 ± 0.1	0.5 ± 0.1	2.8 ± 0.1	4.3 ± 0.1[d]	0.7 ± 0.2	ND	ND	1.6 ± 0.1		

[a] The values were presented as mean ± SE ($n = 3$); n, the number of determinations; ND, nondetectable. [b] $P \leq 0.01$ compared with old leaf. [c] $P \leq 0.05$ compared with Longjing tea (Sansia, Taipei). [d] $P \leq 0.01$ compared with Longjing tea (Sansia, Taipei).

Table 5. Catechin Levels of the Partially Fermented Tea in Taiwan and China

	mg/g of tea[a]								ratio of	
	EGC	C	EC	EGCG	GCG	4″-Me-EGC G	3″-Me-EGC G	ECG	4″-MeEGCG/ total EGCG	3″-MeEGCG/ total EGCG
					Taiwan					
oolong tea	7.0 ± 0.2	ND	1.1 ± 0.0	3.3 ± 0.3	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.5 ± 0.1	0.03	0.06
oolong tea (Tongting)	3.3 ± 0.6	ND	0.7 ± 0.1	2.7 ± 0.4	0.1 ± 0.0	0.1 ± 0.2	0.6 ± 0.0[b]	0.6 ± 0.1	0.04	0.16
high mountain (Ruili)	3.3 ± 0.1	0.1 ± 0.0	0.6 ± 0.0	1.4 ± 0.1[b]	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.07	0.09
Tieh-kuan-yin (Taitung)	6.6 ± 1.8	ND	1.0 ± 0.3	2.7 ± 0.8	0.1 ± 0.0	0.4 ± 0.1[b]	0.3 ± 0.1	0.5 ± 0.1	0.10	0.09
					China					
Fo-Shou-Wang (Chengdu)	3.8 ± 0.2	0.1 ± 0.0	1.1 ± 0.1	1.6 ± 0.4[b]	0.1 ± 0.0	ND	ND	0.6 ± 0.2		
rock tea (Wuuyi)	3.7 ± 0.2	0.1 ± 0.0	1.0 ± 0.1	2.6 ± 0.5	0.7 ± 0.1	ND	0.3 ± 0.0[c]	0.8 ± 0.2		0.01
Fenghuang (Chaoan)	1.9 ± 0.2	0.1 ± 0.0	0.3 ± 0.0	2.7 ± 0.2[c]	0.2 ± 0.0	0.3 ± 0.1[b]	0.7 ± 0.0[b]	0.7 ± 0.1	0.01	0.11

[a] The values were presented as mean ± SE ($n = 3$) n, the number of determinations; ND, nondetectable. [b] $P \leq 0.01$ compared with oolong tea (Taiwan). [c] $P \leq 0.05$ compared with oolong tea (Taiwan).

tea (Taiwan) as compared with those of high mountain tea (Taiwan) and oolong tea (Wuuyi, China).

HPLC Separation of Catechins and O-Methylated EGCG in Black and Puerh Teas. Both black and puerh teas are classified as fully fermented teas. The representative HPLC patterns of Yunnan black tea are illustrated in **Figure 2D**, and the levels of catechins are summarized in **Table 6**. The results show both black tea and puerh tea do not contain detectable O-methylated EGCG.

Suppressing Effects of O-Methylated EGCG on LPS−Induced NO Production in Macrophages. Nitric oxide can function as an immunomodulator. It can inhibit leukocyte adhesion on the vascular endothelial cells in inflammatory processes and can increase activities of natural killer cells or lymphokine-activated killer cells (*27*). Thus, we used a murine macrophage, RAW 264.7 cell line, as a model, to realize the NO-suppressing effect of O-methylated EGCG. Treatment of RAW 264.7 cells with LPS for 16 h stimulated nitric oxide (NO) generation in the culture medium (**Figure 3**). The production of NO in this cell culture was strongly inhibited by the presence 3″-Me-EGCG (10 μM). Similar inhibition of nitrite production was observed after exposure of EGCG or 4″-Me-EGCG in RAW 264.7 cells. A higher concentration (20 μM) was needed for the inhibition of nitrite production by 4′,4″-diMe-EGCG. It appeared that O-methylated EGCG suppressed nitrite production.

Inhibition of Inducible NO Synthase Expression by O-Methylated EGCG in Macrophages. Then, O-methylated EGCG, 3″-Me-EGCG, 4″-Me-EGCG, and 4′,4″-diMe-EGCG were tested to determine whether they affect inducible NO synthase protein in macrophages activated with LPS for 16 h.

Table 6. Catechin Levels of Black and Puerh Tea

	mg/g of tea[a]							
	EGC	C	EC	EGCG	GCG	4″-MeEGCG	3″-MeEGCG	ECG
				Black Tea				
Yunnan, China	ND	0.2 ± 0.1	0.5 ± 0.0	0.3 ± 0.1	0.1 ± 0.1	ND	ND	1.4 ± 0.3
Lipton black tea	0.4 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	1.2 ± 0.1	0.1 ± 0.0	ND	ND	1.4 ± 0.1
				Puerh Tea				
50 years	ND	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	ND	ND	ND	0.9 ± 0.5
34 years	0.1 ± 0.0	0.2 ± 0.1	0.5 ± 0.1	1.3 ± 0.3	0.2 ± 0.1	ND	ND	3.0 ± 0.4
16 years	1.5 ± 0.1	1.0 ± 0.4	2.8 ± 0.7	3.7 ± 0.7	0.3 ± 0.1	ND	ND	4.8 ± 0.9
5 years	3.3 ± 0.3	2.1 ± 0.3	4.7 ± 0.5	10.2 ± 1.2	0.7 ± 0.3	ND	ND	8.7 ± 1.1
2 years	2.4 ± 0.7	1.2 ± 0.9	2.1 ± 1.2	3.3 ± 1.3	0.2 ± 0.2	ND	ND	1.7 ± 1.0
1 year	4.1 ± 0.9	0.9 ± 0.1	2.5 ± 0.3	7.0 ± 1.6	0.6 ± 0.3	ND	ND	3.1 ± 0.6

[a] The values were presented as mean ± SE ($n = 3$); n, the number of determinations; ND, nondetectable.

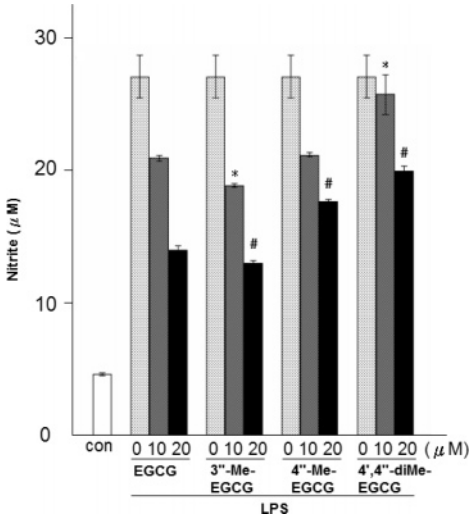


Figure 3. Effects of methylted EGCG on nitrite release in culture medium of LPS-activated macrophages. Each value represents the mean ± SE for three determinations. Significant difference from the control value are indicated as follows: $^{*}P \leq 0.05$ and $^{\#}P \leq 0.01$ compared with the EGCG-treated group.

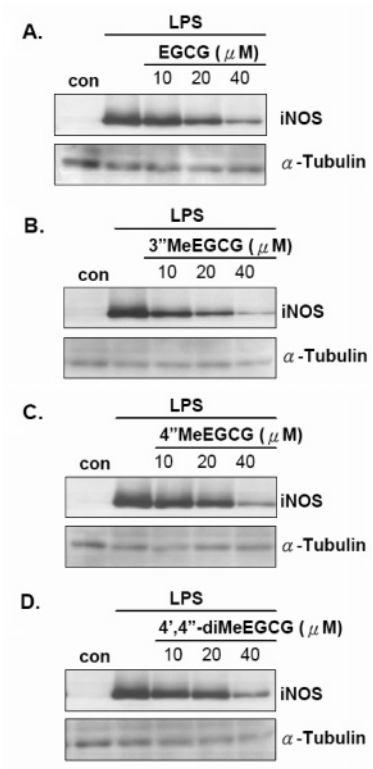


Figure 4. Western blot analysis of the effects of methylated catechins on the inducible NO synthase in LPS-activated macrophages. The experiment was repeated three times with similar results.

At the same concentration of O-methylated EGCG, the inhibitory activity of 3″-Me-EGCG was better than that of EGCG, while the 4′,4″-diMe-EGCG was not **(Figure 4)**. At 20 μM, EGCG and 3″-Me-EGCG inhibited the levels of iNOS protein by 17% and 61%. At 40 μM, EGCG inhibited the levels of iNOS protein by 68% and 3″-Me-EGCG inhibited the levels of iNOS protein completely. The pattern of inhibitory activity was parallel to the generation of NO (**Figure 3**).

DISCUSSION

In the present study, we used isocratic HPLC combined with electrochemical detection (ECD) for the simultaneous determination of catechins and O-methylated EGCG in tea and fresh tea leaf samples. We found that the O-methylated EGCG are naturally present in the tea leaves themselves (**Tables 1** and **2**) and the O-methylated EGCG contents of longjing green tea and oolong teas are higher than that of fresh tea leaves as indicated in **Tables 4** and **5**. This suggests that the manufacturing process of converting the fresh leaves to green tea and oolong tea might increase the level of O-methylated EGCG released from tea leaves when they are extracted. It appears that the O-methylated EGCG biotransformation has continued during the manufacturing processes. Another explanation is that the mechanical operation in the withering process may rupture the outer membrane of the tea leaves, liberating the intracellular O-methylated EGCGs and rendering them available for extraction.

Compared with the publications of others, the catechin levels vary within a much wider range in this study. However, some factors might change the compositions of tea polyphenols. First, the chemical compositions of the tea samples we collected in the local markets when we were traveling in these cities and townships may vary with their species, cultivation conditions, such as weather, temperature, moisture, latitude, and season, and process of manufacturing. It has been established that the levels of polyphenols, especially catechins, are significantly affected by the production process. Thus, the wide-range variation is predicted. Second, because we used water extraction for HPLC analysis, the content of catechins in our current study might be less than that from the ethanol extraction used in previous studies (*28*, *29*). Thus, hot water extraction efficiency might not be complete. Third, the fact that the water of tea leaves does not dry completely might reduce the relative content of

catechins. Furthermore, similar to our previous laboratory data (*30*), the range of amount of EGCG in nonfermented green teas is from 4.31 to 15.27 mg/g of tea in our current data.

EGCG and other catechins undergo substantial metabolism to species including glucuronides, sulfates, and methylated compounds. The biologic activity attributed to EGCG may occur via one or more of its metabolites. Studies suggest that methylation is an important route for EGCG. Recent studies indicate that cytosolic catechol-*O*-methyltransferase (COMT) from liver methylated EGCG to 4″-Me-EGCG and to 4′,4″-diMe-EGCG in rat and human tissues (*31*−*34*). Sano and colleagues (*21*, *22*) demonstrated that O-methyl-EGCG has a stronger antiallergic activity than EGCG does against type I and IV allergies in mice and activation and degranulation in basophilic KU812 cells (*35*). However, the understanding of the biological activities of methylated EGCG is still very limited.

Nitric oxide (NO) is synthesized from L-arginine in the human respiratory tract by enzymes of the NO synthase (NOS) family. In endothelial cells, NO is generated rapidly and transiently at low (picomolar) concentration and induces fast and transient responses in target cells such as smooth muscle cells (*23*). By contrast, in immune and inflammatory responses, cytokines and/or bacterial lipopolysaccharide induced expression of the inducible form of NO synthase (iNOS) leading to relatively slow, sustained, and high-level production of NO (*23*, *25*). In asthma, the increased levels of exhaled NO have a predominantly lower airway origin (*36*, *37*) and appear to be associated with increased expression of corticosteroid-sensitive iNOS (*24*). Oh et al. (*38*) investigated the role of nitric oxide synthases in a mucosa model. They found inducible nitric oxide synthase had a faint expression in control group mice but increased after allergic sensitization, and the amount of inducible nitric oxide synthase was elevated in allergic mice compared with that of the control group. Thus, the evidence suggests that inhibition of iNO would be a useful therapeutic strategy in asthma (*39*). Our current study shows that the inhibition of inducible NO synthase protein level is in the following order: 3″-Me-EGCG > EGCG > 4″-Me-EGCG > 4′,4″-diMe-EGCG. We suggest that the O-methylated EGCG might act as an alternative candidate of antiinflammatory medicine in asthma.

For in vivo antioxidative activity of EGCG, the structural modification of EGCG in the body should be taken into account in addition to its bioavailability. With respect to the structural modification, some EGCG metabolites are expected to exert almost the same antioxidative effect as intact EGCG, but 4′,4″-diMe-EGCG will probably show only a slight activity. This is because the strong antioxidative activity of EGCG is maintained at least by the presence of an *o*-trihydroxyl group or an *o*-dihydroxyl group in either the B ring or the galloyl moiety, whereas modifications at the 4′ and 4″ positions of EGCG, that is, the lack of both the *o*-trihydroxyl group and the *o*-dihydroxyl group in its structure, result in loss of the antioxidative activity (*40*, *41*). That the inhibition of iNOS by the 3″-Me-EGCG is better than by EGCG might involve structure−activity relationships. However, we need further studies to elucidate the relationship between the structure of conjugated EGCG metabolites and their iNOS suppression efficacy. To study the details of the effects, the energy or distribution of electrons in EGCG must be investigated. Especially, the role of the O-methyl group in the galloyl group of 3″-Me-EGCG must be clarified in further study before application of 3″-Me-EGCG to antiinflammatory medicine in allergy response.

Our results show the increasing significance of the potentially beneficial role of tea catechins in human health, and the metabolic fate of tea catechins in the body have recently become a subject of considerable interest. Based on our results, we suggest that O-methylated EGCG in green tea could play a favorable role in our daily lives in the prevention of a number of diseases including the inflammation of asthma. Further study will focus on the biological activities of O-methylated EGCG and determining the molecular mechanism by which O-methylated EGCG inhibits nitric oxide induction. In conclusion, the finding of the present study indicates that daily intake of tea drinks, especially longjing tea infusion, is thought to have the potential to prevent inflammation of the respiratory tract.

ACKNOWLEDGMENT

We thank Professor M. Sano for his generous gift of methylated catechins and stimulating discussion. We also thank Chen-Sheng Lee, Yu-Jen Tsai, and Jui-Lin Hsu for their kindness in supplying tea samples. The critical reading and annotation of this paper by Professor Russell J. Molyneux is highly appreciated.

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Received for review April 1, 2005. Revised manuscript received June 30, 2005. Accepted July 7, 2005. This study was supported by the National Science Council, NSC-93-2211-B-002-001, NSC-93-2320-B-002-111, and NSC-93-2320-B-002-127.

JF0507442