Identification of Biliary Metabolites of (−)-Epigallocatechin Gallate in Rats

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After oral administration of (−)-epigallocatechin gallate (EGCg) to rats, its biliary metabolites were examined. Although a large part of the biliary metabolites was found to exist in conjugated forms, it was difficult to separate the conjugated forms. Thus the free form of biliary metabolites was prepared by *β*-glucuronidase/sulfatase treatment and was purified by HPLC. Six compounds purified were subjected to FAB−MS and NMR analyses. The six metabolites thus obtained were shown to be EGCg, 3′-*O*-methyl-EGCg, 4′-*O*-methyl-EGCg, 3″-*O*-methyl-EGCg, 4″-*O*-methyl-EGCg, and 4′,4″-di-*O*-methyl-EGCg, respectively. The six EGCg metabolites and their conjugates excreted during a 4-h period were estimated to be roughly 0.1% and 3.3% of the administered EGCg, respectively. In addition, 4″-*O*-methyl-EGCg and 4′,4″-di-*O*-methyl-EGCg were estimated to exist only in the sulfate form, but the other four metabolites existed in both glucuronide (and/or sulfoglucuronide) and sulfate forms.

Keywords: *(−)-Epigallocatechin gallate; catechin; tea; glucuronidase/sulfatase; biliary metabolite*

INTRODUCTION

Recent epidemiological studies have revealed that the intake of flavonoids is inversely associated with the risk of coronary heart disease and stroke (Hertog et al., 1993, 1995; Keli et al., 1996). These studies have suggested that tea catechins as the major ingestible flavonoids may be responsible for this inverse association. Imai et al. (1997) have reported that a negative association between green tea consumption and cancer incidence was found in a prospective cohort study of a Japanese population. They also suggested that the presence of tea catechins, especially (−)-epigallocatechin gallate (EGCg), may be an important factor for this negative association. With the increasing significance of the potentially beneficial role of tea catechins in human health, the metabolic fate of tea catechins in the body has recently become a subject of considerable interest. In particular, attention has been focused on the metabolism of EGCg because EGCg is the most abundant catechin (about 50% of tea catechins) in green tea and has stronger physiological activities than the other tea catechins. While there have been some reports with respect to the absorption, distribution, and excretion of EGCg (Lee et al., 1995; Okushio et al., 1995; Nakagawa and Miyazawa, 1997), little attention has been given to determining the chemical structures of EGCg metabolites formed in the body.

We have recently reported the identification of (−)-epicatechin metabolites in rat urine (Okushio et al., 1999a) and methylation of tea catechins including EGCg by rat liver homogenates (Okushio et al., 1999b). The present study was conducted to determine the chemical structures of EGCg metabolites in rat bile after oral administration of EGCg.

MATERIALS AND METHODS

Chemicals and Reagents. EGCg was purchased from Kurita Chemical Co. (Tokyo, Japan). *β*-Glucuronidase type H-1 from helix pomatia (G 0751) and D-saccharic acid 1,4-lactone were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were available products of analytical grade or HPLC grade.

Animals and Animal Experiments. Wistar male rats (6 weeks of age, 180−210 g, specific pathogen free) were purchased from Charles River Japan, Inc., and were maintained on a special diet free from naturally occurring polyphenols for a week (Okushio et al., 1996). Bile duct cannulation was carried out with the animals according to the method of Hackett et al. (1979). EGCg (100 mg) in 1 mL of 10% aqueous ethanol was administered orally to the rats by direct gastric intubation. Bile samples from 20 rats were collected in test tubes containing 3 mL of 0.1 M sodium acetate buffer (pH 5.0) over a 4-h period after administration. All samples were frozen immediately and stored at −20 °C until use.

Purification of EGCg Metabolites from Bile. The bile sample (about 100 mL) was washed three times with the same volume of ethyl acetate. The aqueous phase was evaporated to dryness, and the residue was dissolved in 100 mL of 0.1 M sodium acetate buffer (pH 5.0) containing 1% ascorbic acid and 0.15 mM ethylenediaminetetraacetic acid (EDTA). To the solution was added *β*−glucuronidase type H-1 (400 mg) which is a mixture of *β*-glucuronidase (140 000 units) and sulfatase (7000 units). The reaction mixture was incubated at 37 °C for 2 h with gentle shaking. The reaction mixture was extracted three times with the same volume of ethyl acetate. The organic phase was concentrated to dryness. The residue was dissolved in acetonitrile/ethyl acetate/water (12:0.6:90 by volume) and was subjected to preparative HPLC. The preparative HPLC was performed with a Capcell Pak C18 AG120 (Shiseido Co., Ltd., 20 mm i.d. × 250 mm) in a Jasco liquid chromatograph apparatus equipped with a Jasco UV-970 detector. Elution was done with acetonitrile/ethyl acetate/water (12:0.6:90 by volume) at a flow rate of 8 mL/min at 40 °C. The elution pattern was monitored by measuring the absorbance at 280 nm. Four fractions were collected as shown in Figure 1A, concentrated to remove the organic solvents, and then freeze-dried. Four major peaks were found to be present in fraction 2, and one of the four peaks was detected in rats who were not administered EGCg. Hence, three peaks were further purified by semipreparative HPLC (Figure 1B). The semipreparative HPLC was carried out with an analytical column of Capcell Pak C18

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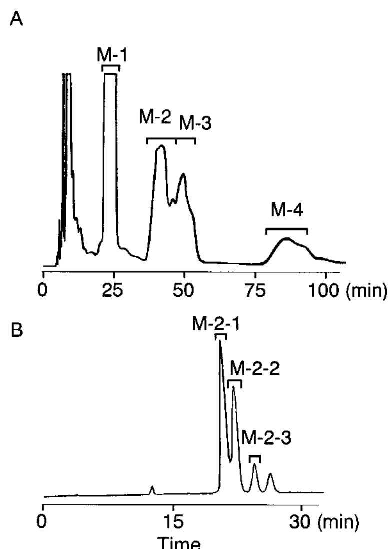

Figure 1. (A) Preparative HPLC profile showing separation of (−)-epigallocatechin gallate metabolites in the free form from rat bile. (B) Semipreparative HPLC profile showing further purification of M-2 fraction obtained from preparative HPLC. Conditions of preparative HPLC and semipreparative HPLC were described in Materials and Methods.

AG120 (Shiseido Co., Ltd., 4.6 mm i.d. × 250 mm) in the same Jasco HPLC system as mentioned above. The HPLC conditions were the same as those of the preparative HPLC except for the use of a flow rate of 0.8 mL/min. Finally, six purified compounds were obtained and were analyzed by analytical HPLC, LC-MS, and NMR. Analytical HPLC was carried out with a Capcell Pak C18 AG120 in a Waters liquid chromatograph apparatus equipped with a Waters model M996 photodiode array detector. The column was eluted with acetonitrile/ethyl acetate/water (12:0.6:90 by volume) at a flow rate of 0.8 mL/min at 40 °C. The elution pattern was monitored by measuring the absorbance at 280 nm, and UV spectra of the compounds were also measured by using wavelengths in the range of 200−400 nm.

Analysis of the EGCg Metabolites in Bile. Bile samples (12.5 mL) were collected from four rats 0−4 h after administration of EGCg (100 mg/rat). The bile sample was extracted three times with 12 mL of ethyl acetate, and the extract was evaporated to dryness. The water phase was also evaporated to dryness and then was dissolved in 1.2 mL of 0.1 M sodium acetate buffer (pH 5.0) containing 1% ascorbic acid and 0.15 mM EDTA and then divided into three portions. Each portion (0.35 mL) was mixed separately with 0.15 mL of β-glucuronidase type H-1 (2600 units of β-glucuronidase, 130 units of sulfatase) dissolved in 0.1 M sodium acetate buffer (pH 5.0), 0.15 mL of the β-glucuronidase type H-1 solution containing 100 mM D-saccharic acid 1,4-lactone (β-glucuronidase inhibitor), and 0.15 mL of 0.1 M sodium acetate buffer (pH 5.0). The mixture was incubated at 37 °C for 5 h. The reaction mixture was added to 1.5 mL of ethanol, and the resulting precipitate was removed by centrifugation at 15 000*g* for 10 min at 0 °C. The supernatant was evaporated to dryness. The residue was dissolved in 0.1 mL of 10% aqueous acetonitrile and was subjected to the analytical HPLC as described above. Quantitative determination of EGCg metabolites in the free form was estimated with a standard curve of EGCg. Conjugation ratio (percentage) of glucuronide (or sulfoglucuronide form) and sulfate forms of each EGCg metabolite were also estimated by the following equation:

$$\text{glucuronide (and/or sulfoglucuronide) form (\%)} = 100(A - B)/A$$

$$\text{sulfate form (\%)} = 100B/A$$

where A is the amount of each EGCg metabolite formed by treating with β-glucuronidase type H-1 and B is the amount of each EGCg metabolite formed by treating with β-glucuronidase type H-1 in the presence of D-saccharic acid 1,4-lactone.

LC-MS and NMR Analyses. LC-MS (FAB ionization method) analysis was carried out with a Jasco HPLC system (900 series) coupled to a JEOL JMS DX-300 mass spectrometer. The HPLC conditions were the same as those of the analytical HPLC as described above. NMR spectra were recorded on a JEOL lambda-500 system. Samples (1−5 mg) were dissolved in 0.5 mL of deuterated acetone (acetone-d_6) (Merck, Germany). Chemical shifts are expressed in ppm relative to tetramethylsilane (TMS) as an internal standard.

RESULTS

Structural Analysis of EGCg Metabolites. In this study, we tried to determine structures of EGCg metabolites in rat bile after oral administration of EGCg because urinary metabolites having the EGCg skeleton were not detected in the rats. Six EGCg metabolites were purified from the rat bile after enzymatic treatment with β-glucuronidase type H-1, which has both β-glucuronidase and sulfatase activities. First, three purified compounds (M-1, M-3, and M-4) were obtained by preparative HPLC (Figure 1A). Then, the M-2 fraction was purified by semipreparative HPLC (Figure 1B), and three purified compounds (M-2-1, M-2-2, and M-2-3) were further obtained.

Structural analysis of each compound was performed by HPLC, MS, and UV and NMR spectroscopy. The compound M-1 showed the same retention time and UV spectrum as authentic EGCg, and its ^{1}H NMR data were superimposable with those of EGCg. Also, it was found that the retention time and UV and mass spectrum of M-2-1 were the same as those of 4″-*O*-methyl-EGCg reported in our previous paper (Okushio et al., 1999b). ^{1}H NMR spectrum of M-2-1 was shown to be in accord with that of 4″-*O*-methyl-EGCg. As a result, the compounds M-1 and M-2-1 were identified as EGCg and 4″-*O*-methyl-EGCg, respectively.

Negative FAB−MS data of M-2-2 showed a $[M-H]^-$ ion peak at m/z 471, corresponding to monomethylated EGCg. ^{1}H NMR spectrum of M-2-2 was very similar to that of EGCg, except for two proton signals from H-2″ (δ 7.03) and H-6″ (δ 7.16) and a methoxy signal (δ 3.79) being observed. In addition, nuclear Overhauser effect (NOE) was observed between the methoxy protons and H-2″. Thus, the compound M-2-2 was identified as 3″-*O*-methyl-EGCg.

A pseudomolecular ion peak of M-2-3 at m/z 471 $[M-H]^-$ in negative FAB−MS was in agreement with that of monomethylated EGCg. ^{1}H NMR spectrum of M-2-3 was found to be similar to that of EGCg, but two separate signals derived from H-2′ (δ 6.71) and H-6′ (δ 6.72) protons and the appearance of a methoxy signal (δ 3.66) were observed. The H-2″ and H-6″ protons of EGCg and 4′-*O*-methyl-EGCg (Table 1) showed the same chemical shift whereas the two protons of 3″-*O*-methyl-EGCg were separated from each other. This separation was regarded as being due to the replacement of the hydroxyl group at 3″ position of EGCg by a methoxy group. On the basis of the above consideration, the fact that H-2′ and H-6′ protons which have the same chemical shift in EGCg were observed as two different signals in M-2-3 indicated that a methyl group was located at 3′ position. As a result, M-2-3 was identified as 3′-*O*-methyl-EGCg.

Negative FAB−MS data of M-3 showed a $[M-H]^-$ ion peak at m/z 471, in agreement with monomethylated

Table 1. ^{1}H NMR Chemical Shifts[a] of (−)-Epigallocatechin Gallate Metabolites in Rat Bile

	4″-*O*-methyl-EGCg (M-2-1)	3″-*O*-methyl-EGCg (M-2-2)	3′-*O*-methyl-EGCg (M-2-3)	4′-*O*-methyl-EGCg (M-3)	4′,4″-di-*O*-methyl-EGCg (M-4)
H-2	5.07 s	5.08 s	5.09 s	5.08 s	5.09 s
H-3	5.51 m	5.46 m	5.51 m	5.47 m	5.49 m
H-4a[b]	2.92 dd (2.0, 17.0)	2.96 dd (2.8, 17.4)	2.94	2.93 d(17.3)	2.93 dd(2.0, 17.7)
H-4b[b]	3.03 dd (4.4, 17.0)	3.04 dd (4.5, 17.4)	3.03	3.03 dd (4.7, 17.3)	3.04 dd (4.6, 17.7)
H-6	6.03 d (2.5)	6.03 d (2.5)	6.04 d (2.5)	6.03 d (2.3)	6.03 d (2.1)
H-8	6.01 d (2.5)	6.02 d (2.5)	6.02 d (2.5)	6.02 d (2.3)	6.02 d (2.1)
H-2′	6.64 s	6.64 s	6.71 d (2.0)	6.68 s	6.72 s
H-6′	6.64 s	6.64 s	6.72 d (2.0)	6.68 s	6.72 s
H-2″	7.01 s	7.03 d (1.8)	7.04 s	7.05 s	7.03 s
H-6″	7.01 s	7.16 d (1.8)	7.04 s	7.05 s	7.03 s
OCH_3	3.81 s	3.79 s	3.66 s	3.73 s	3.73 s, 3.81 s

[a] Chemical shifts are expressed in ppm downfield from the signal for TMS in acetone-d_6 and coupling constants in Hertz are in parentheses. [b] H-4a and H-4b were provisionally determined.

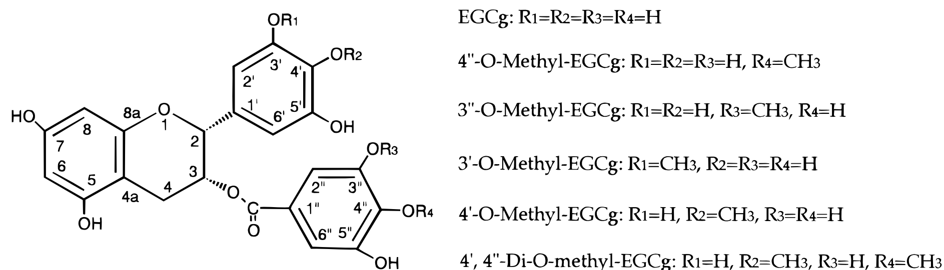

Figure 2. Structures of biliary metabolites of (−)-epigallocatechin gallate formed by glucuronidase/sulfatase mixture.

EGCg. The ^{1}H NMR spectrum of M-3 was analogous to that of 4″-*O*-methyl-EGCg, but the chemical shift (δ 3.73) of methoxy signal of M-3 was observed to be different from that of 4″-*O*-methyl-EGCg (δ 3.81). Heteronuclear multiple-bond connectivity (HMBC) experiments showed a cross-peak between C-4′ and methoxy protons. From these results, the compound M-3 was identified to be 4′-*O*-methyl-EGCg. The assignment of ^{13}C NMR spectrum was as follows: ^{13}C NMR (acetone-d_6, 125 MHz) δ 26.5 (C-4), 60.4 (methoxy carbon), 69.3 (C-3), 78.1 (C-2), 95.8 (C-8), 96.4 (C-6), 106.8 (C-2′, 6′), 109.8 (C-2″, 6″), 125.5 (C-1″), 126.8 (C-1′), 135.3 (C-4′), 146.2 (C-3″, 5″), 151.1 (C-3′, 5′), 157.0 (C-8a), 157.7 (C-5, 7), 166.0 (C=O). Signals of C-4a and C-4″ were not observed because of the small quantity of M-3.

In the case of M-4, a pseudomolecular ion peak at *m*/*z* 485 $[M-H]^-$ was observed by negative FAB−MS, suggesting that M-4 is dimethylated EGCg. ^{1}H NMR spectrum of M-4 was very similar to that of EGCg, except for the appearance of two methoxy signals. The chemical shift of one methoxy signal (δ 3.73) was in accord with that of 4′-*O*-methyl-EGCg and the shift of another signal (δ 3.81) agreed with that of 4″-*O*-methyl-EGCg. From these observations, the compound M-4 was judged to be 4′,4″-di-*O*-methyl-EGCg. Full assignments of the ^{1}H NMR signals of M-2-1, M-2-2, M-2-3, M-3, and M-4 are shown in Table 1, and the structures of the six metabolites of EGCg identified in this study are illustrated in Figure 2.

Quantitative Analysis of EGCg Metabolites in Bile. After oral administration of EGCg (100 mg/rat), biliary metabolites of EGCg in both the free and the conjugated forms were determined by the analytical HPLC (Table 2). Only EGCg and 4″-*O*-methyl-EGCg were present in the free form, and their amounts (0.12% of dosing) were very low. On the other hand, the total amount of the six metabolites detected in the conjugated forms was found to be 3.28% of dosage. Of this, EGCg in the conjugated forms was predominant with an amount of 2.65% of dosing. The other five methylated EGCg's in the conjugated forms were in the range of 0.06−0.25% of dosing. Total recoveries of the EGCg metabolites in the free and conjugated forms were estimated to be 3.40% of oral administration.

Table 2. Recoveries of (−)-Epigallocatechin Gallate Metabolites Excreted in Rat Bile

metabolites	biliary excretion (% of dose) free form[a]	conjugated form
EGCg	0.07	2.65
4″-*O*-methyl-EGCg	0.05	0.25
3″-*O*-methyl-EGCg	ND	0.11
4′-*O*-methyl-EGCg	ND	0.11
3′-*O*-methyl-EGCg	ND	0.10
4′,4″-*O*-methyl-EGCg	ND	0.06
total	0.12	3.28

[a] ND = not detected.

Table 3. Proportions of Glucuronide (and/or Sulfoglucuronide) and Sulfate Forms in the Conjugated (−)-Epigallocatechin Gallate Metabolites

conjugated EGCg metabolites	glucuronide (or sulfoglucuronide) form (%)	sulfate form (%)
EGCg	58.1	41.9
4″-*O*-methyl-EGCg	0	100
3″-*O*-methyl-EGCg	74.8	25.2
3′-*O*-methyl-EGCg	73.9	26.1
4′-*O*-methyl-EGCg	76.1	23.9
4′,4″-*O*-methyl-EGCg	0	100

Proportions of Glucuronide (and/or Sulfoglucuronide) and Sulfate Forms in the Conjugated EGCg Metabolites. After the bile sample was washed with ethyl acetate, the water phase was treated with glucuronidase/sulfatase mixture (β-glucuronidase type H-1) in the absence or presence of β-glucuronidase inhibitor (D-saccharic acid 1,4-lactone). Then, EGCg and its methylated derivatives formed were extracted with ethyl acetate and were determined by the analytical HPLC. Table 3 shows the proportions of glucuronide (and/or sulfoglucuronide) and sulfate forms in each

EGCg metabolite. The conjugated EGCg was present in the glucuronide (and/or sulfoglucuronide) form (about 60%) followed by the sulfate form (about 40%). 3′-*O*-Methyl-EGCg, 4′-*O*-methyl-EGCg, and 3″-*O*-methyl-EGCg were present in the glucuronide (and/or sulfoglucuronide) form (about 75%) and in the sulfate form (about 25%). Interestingly, both 4″-*O*-methyl-EGCg and 4′,4″-di-*O*-methyl-EGCg were found to be in the sulfate form, and their glucuronide forms were not detected at all by the analytical method used in this study.

DISCUSSION

We have already reported that EGCg itself is absorbed into the portal vein after oral administration to rats (Okushio et al., 1995). It was reported that ingested EGCg was detected in rat plasma (Unno and Takeo, 1995; Chen et al., 1997; Nakagawa and Miyazawa, 1997) and human plasma (Lee et al., 1995; Unno et al., 1996; Nakagawa et al., 1997; Yang et al., 1998). Further, Lee et al. (1995) reported that EGCg detected in human plasma was mostly in the glucuronide and sulfate forms, but EGCg and its conjugates were hardly found in human urine. Further, neither the free nor conjugated forms of EGCg were detected in rat urine in our preliminary experiments. From the above observations, it was supposed that EGCg and its conjugates were excreted in the bile. Thus, we examined EGCg metabolites in rat bile. First, the bile sample was washed with ethyl acetate and subjected to HPLC analysis in an attempt to separate the conjugated forms of EGCg metabolites. However, the HPLC chromatogram showed that biliary components were hardly retained to the column and were eluted within 5 min without separation. On the other hand, after enzyme treatment of the bile sample with β-glucuronidase/sulfatase mixture, five compounds whose UV spectra were very similar to those of EGCg were detected in addition to a substantial amount of EGCg. Accordingly, our attempts in this study were directed toward determining chemical structures of the six EGCg metabolites produced by the enzyme mixture. The EGCg metabolites were purified and were finally identified as EGCg, 3′-*O*-methyl-EGCg, 4′-*O*-methyl-EGCg, 3″-*O*-methyl-EGCg, 4″-*O*-methyl-EGCg, and 4′,4″-di-*O*-methyl-EGCg. Prior to this study, we demonstrated that 4″-*O*-methyl-EGCg was also produced by the enzymatic reaction with EGCg and rat liver homogenate in the presence of (*S*)-adenosyl-L-methionine (Okushio et al., 1999b). These findings indicate that methylation is one of the most important metabolic routes of EGCg.

Each amount of the six EGCg metabolites (EGCg and its methylated derivatives) identified here and their conjugates was examined. The total amount of EGCg and its methylated derivatives was only about 3.7% of those presented in the conjugated forms. These results suggest that almost all EGCg absorbed undergoes conjugation such as glucuronidation and sulfation in the intestine and the liver. Total recoveries of EGCg, its methylated derivatives, and their conjugates excreted in the bile were estimated to be about 3.4% of the ingested EGCg. EGCg and its methylated derivatives were hardly detected in the urine and in the bile collected at 4−8 h period even after the enzyme treatment (data not shown). On the basis of these observations, at least 3.4% of the administered EGCg was absorbed in the body. On the other hand, it has been found that EGCg was detected in rat portal blood (Okushio et al., 1995) but not in venous blood from rat tail in this study (data not shown). These results seem to imply that a large part of EGCg absorbed via the portal blood undergoes first-pass effect due to hepatic elimination and is excreted into the bile prior to entering systemic circulation. Chen et al. (1997) detected EGCg in rat plasma by using a HPLC−coulchem electrode array system, which is more sensitive than the HPLC−UV detection system used in this study, and they reported that the bioavailabilities of EGCg intragastrically given as pure EGCg and as decaffeinated green tea to rats were estimated to be 1.6% and 0.1%, respectively, of ingested EGCg. These values of the bioavailability also appear to be less than those of the biliary excretion of EGCg metabolites (3.4% of dosing) estimated in this study, supporting the substantial first-pass hepatic elimination as described above.

We found that except for 4″-*O*-methyl-EGCg and 4′,4″-di-*O*-methyl-EGCg, EGCg and its methylated metabolites underwent glucuronidation (and/or sulfoglucuronidation) or sulfation. However, 4″-*O*-methyl-EGCg and 4′,4“-di-*O*-methyl-EGCg were observed to be only in their sulfate forms. The reason for the observation is unclear. One possible explanation is either that EGCg conjugated with glucuronic acid in the intestinal mucosa or liver is not susceptible to methylation at its 4″ position in the liver, or that methylation of EGCg at 4″ position is resistant to the glucuronidation in the liver.

It is believed that the potent antioxidative ability of EGCg may be related closely to its physiological activity. In fact, EGCg and other tea catechins were found to prevent low-density lipoprotein from oxidation in vitro (Ishikawa et al., 1997). However, in vivo antioxidative activity of EGCg should be evaluated taking into account its structural modification in the body in addition to its bioavailability. With respect to the structural modification, five of the six EGCg metabolites identified here are expected to exert almost the same antioxidative effect as intact EGCg, but 4′,4″-di-*O*-methyl-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 ortho-trihydroxyl group or an ortho-dihydroxyl group in either the B ring or the galloyl moiety, whereas modification at the 4′ and 4″ positions of EGCg, that is, the lack of both the ortho-trihydroxyl group and the ortho-dihydroxyl group in its structure, result in loss of the antioxidative activity (Nanjo et al., 1996 and 1999). However, without further study on the structural elucidation of conjugated EGCg metabolites, the *in vivo antioxidative* efficacy of EGCg cannot fully be comprehended.

ABBREVIATIONS USED

EGCg, (−)-epigallocatechin gallate; EDTA, ethylenediaminetetraacetic acid; TMS, tetramethylsilane.

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