RESEARCH ARTICLE

Binding affinity of tea catechins for HSA: Characterization by high-performance affinity chromatography with immobilized albumin column

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Catechins are the major polyphenols in green tea leaves. Recent studies have suggested that the catechins form complexes with HSA for transport in human blood, and their binding affinity for albumin is believed to modulate their bioavailability. In this study, the binding affinities of catechins and their analogs were evaluated and the relationship between the chemical structure of each catechin and its binding property were investigated. Comparing these catechins by HPLC analysis with the HSA column, we showed that galloylated catechins have higher binding affinities with HSA than non-galloylated catechins. In addition, pyrogallol-type catechins have a high affinity compared to catechol-type catechins. Furthermore, the binding affinity of the catechin with 2,3-*trans* structure was higher than those of the catechin with 2,3-*cis* structure. The importance of the hydroxyl group on the galloyl group and B-ring was confirmed using methylated catechins. These results indicate that the most important structural element contributing to HSA binding of tea catechins is the galloyl group, followed by the number of hydroxyl groups on the B-ring and the galloyl group or the configuration at C-2. Our findings provide fundamental information on the relationship between the chemical structure of tea catechins and its biological activity.

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1 Introduction

Tea is one of the most popular beverages consumed worldwide. Tea contains many compounds, especially polyphenols, and epidemiological studies show that polyphenolic compounds present in tea reduce the risk of a variety of diseases [1–3]. Their diverse biological activities have been attributed to a group of polyphenol compounds, namely catechins present in green tea leaves [4]. The major catechins of green tea extract are (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), and (−)-epigallocatechin gallate (EGCg) (Fig. 1). These catechin with 2,3-*cis* structure (*cis*-type catechin) have been found to act as free radical scavengers and have been widely studied for their antioxidant

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Abbreviations: (+)-C, (+)-catechin; **(−)-C,** (−)-catechin; **cis-type catechin,** catechin with 2,3-cis structure; **Cg,** (−)-catechin gallate; **EC,** (−)-epicatechin; **ECg,** (−)-epicatechin gallate; **EGC,** (−)-epigallocatechin; **EGCg,** (−)-epigallocatechin gallate; **EGCg-3"OMe,** (−)-epigallocatechin-3-(3"-O-methyl) gallate; **EGCg-4"OMe,** (−)-epigallocatechin-3-(4"-O-methyl) gallate; **EGCg-4′OMe,** (–)-4′-O-methylepigallocatechin-3-gallate; **GC,** (−)-gallocatechin; **GCg,** (−)-gallocatechin gallate; **trans-type catechin,** catechin with 2,3-trans structure; **67LR,** 67 kDa laminin receptor

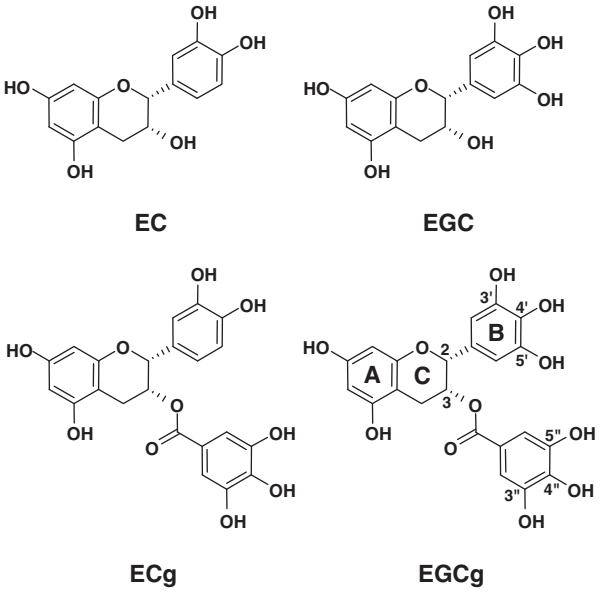


Figure 1. Chemical structures of *cis*-type catechins.

activity *in vitro*. The number of the hydroxyl groups on the B-ring and the presence or absence of a galloyl moiety result in the differences in the structures of these catechins.

The hydroxyl group on the B-ring is the most significant factor in the scavenging reactive oxygen species. Catechins with a pyrogallol-type structure on the B-ring, such as EGC and EGCg, have strong antioxidant activities [5]. On the other hand, many experiments have shown that the catechins with a galloyl moiety, such as ECg and EGCg, have more effective biological activities than their homologues lacking the galloyl group, including growth inhibition of human lung cancer cell line: ECg > EGCg > EGC > EC [6]; inactivation effects on human type-A influenza virus, ECg > EGCg > > EGC [7]; inhibitory effects on the oxidative modification of LDL, EGCg > ECg > EGC > EC or EGCg > ECg > > EC > EGC [8, 9]. However, concerns of their *in vivo* activity, especially regarding their absorption, metabolism, and bioavailability remain unresolved.

HSA, the most abundant protein in blood, is a 66 kDa protein that plays an important role in the reversible binding of many compounds such as free fatty acids, steroids, and some metals and drug metabolites. The binding ability of HSA in blood is an important factor in the transport and release of various endogenous and exogenous compounds [10]. Protein complexes of these compounds may replenish free compounds removed by metabolic processes. Therefore, the interaction with HSA directly influences transportation and metabolism of the compounds, and consequently contributes to the magnitude of their biological activities *in vivo*.

Several recent studies have suggested that the catechins form complexes with albumin for transport in human blood, and in this study, their binding affinity to albumin is believed to modulate their bioavailability. Direct methods using native-PAGE/Western blotting followed by redox-cycling staining indicated that EGCg non-covalently binds to HSA in human serum after incubation with EGCg [11]. Indirect methods based on the quenching of intrinsic tryptophan fluorescence indicated that EGCg non-covalently binds to HSA or BSA through hydrogen bonding or hydrophobic interactions under reductive conditions [12, 13]. In addition, it was indicated that ECg shows high binding affinity for serum albumin in comparison with (+)-catechin ((+)-C), EC, or EGC [14]. These results suggest that the galloyl moiety is important in the interaction of catechins with serum albumin. Although the binding capacity for HSA also contributes to the stabilization of EGCg in human serum [11], the relationship between the chemical structure of catechins and its binding affinity for HSA has not yet been characterized in detail.

In the present study, the binding affinities of the *cis*-type catechin (EC, ECg, EGC, and EGCg) and their analogs such as catechin with 2,3-*trans* structure (*trans*-type catechin) and methylated catechins were evaluated and the relationship between the chemical structure of each catechin and its binding affinity were investigated.

2 Materials and methods

2.1 Materials

EC, ECg, EGC, EGCg, (+)-C, (−)-catechin ((−)-C), (−)-catechin gallate (Cg), (−)-gallocatechin (GC), and (−)-gallocatechin gallate (GCg) were kindly provided by Mitsui Norin (Shizuoka, Japan). (−)-Epigallocatechin-3-(3"-O-methyl) gallate (EGCg-3"OMe), (−)-epigallocatechin-3-(4"-O-methyl) gallate (EGCg-4"OMe), and (−)-4'-O-methyl-epigallocatechin-3-gallate (EGCg-4′OMe) were synthesized according to the method previously reported [15, 16]. HSA was purchased from Sigma (St. Louis, MO, USA). All other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2 HPLC analysis with immobilized HSA

Affinity chromatography on an immobilized HSA column was performed as previously described with modifications [17]. Briefly, catechins were prepared in MeOH (100 μM) and then injected (injection volume 10 μL). Separation of the catechins were carried out on a Gulliver system (Jasco, Tokyo, Japan) with a UV detector, using a Sumichiral HSA column (Sumika Chemical Analysis Service, Osaka, Japan) with a mobile phase consisting of 20% ACN with 100 mM phosphate buffer (pH 5.0). The flow rate was 0.9 mL/min and the column temperature was controlled at 37°C. The elution profiles were monitored by absorbance at 200 nm. All statistical analyses were performed using SPSS software version 14.0 for Windows. Data were analyzed by multiple comparisons (Tukey's multiple-range test) at the each criterion of 1% level ($p < 0.01$).

2.3 Protein binding experiment by ultrafiltration

Catechins (100 μM) were incubated in 500 μL of 200 mM sodium phosphate buffer (pH 6.4) containing ascorbic acid (10 mM) without (control) or with 10 mg/mL HSA at 37°C for 10 min. The samples were subjected to centrifugal filtration using Microcon-30 (molecular weight cut-off of 30 000 Da; Millipore, Bedford, MA, USA) to obtain filtrate. The filtrate was analyzed for the amount by HPLC. The amount of catechins was evaluated by a RP-HPLC analysis as previously reported [11]. Briefly, separation of the catechins was carried out on a Gulliver system with a UV detector, using a Capcell Pak C18 UG120 column (Shiseido, Tokyo, Japan) with a mobile phase consisting of 20 or 30% methanol with water with 0.1% TFA. The flow rate was 1 mL/min, and the elution profiles were monitored by absorbance at 285 nm. Protein binding (%) was calculated as previously reported [18]. Statistical analysis by Tukey's multiple-range test was used to evaluate the significant differences of the data at the each criterion of 1% level ($p < 0.01$).

3 Results

3.1 Binding affinity of *cis*-type catechins for HSA

Several reports using spectrofluorometry, crystallography, and affinity electrophoresis have indicated that green tea catechins bind to albumin [12–14, 19, 20]. However, the relationship between the chemical structures of catechins and their binding affinity for HSA has not yet been characterized in detail. To examine the binding affinity of tea catechins for HSA, the interaction of *cis*-type catechins (EC, ECg, EGC, and EGCg) with HSA was investigated by HPLC analysis with an immobilized HSA column, and the method was compared with the conventional ultrafiltration method. This affinity HPLC method has advantages over other traditional, solution-based methods, including high precision, automation, speed, and good correlation [17]. For this method, the HSA partition coefficient (K_{HSA}) for a compound can be calculated as follows: $K_{HSA} = (t_R - t_0)/t_0$, where t_R is the retention time of the compound and t_0 is the retention time of the unretained marker compound (citrate in this case). It has been reported that K_{HSA} is correlated with equilibrium dialysis and ultrafiltration method. From the results of four *cis*-type catechins (EC, ECg, EGC, and EGCg) by HPLC method, their K_{HSA} values were related as follows: EGCg > ECg > > EGC > EC (Fig. 2 and Table 1). Among the four catechins, ECg and EGCg (which possess a galloyl moiety) displayed larger K_{HSA} values than EC or EGC (which lack the galloyl moiety). This is correlated with ultrafiltration method. Furthermore, the binding affinities of pyrogallol-type catechins (EGC and EGCg) were higher than those of catechol-type catechins (EC and ECg) in the evaluation of K_{HSA} values. However, despite the difference of the number of hydroxyl groups on the B-ring between pyrogallol-type catechins and catechol-type catechins, no difference in binding ratio was found in the evaluation by ultrafiltration method. These comparison results suggest that affinity HPLC method is advantageous in that it has high selectivity for evaluation of the binding

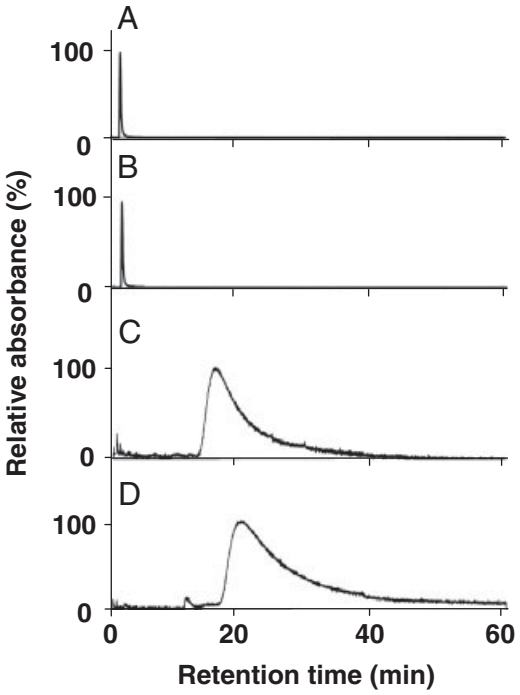


Figure 2. HPLC analysis of catechins with HSA column. Catechins (100 μM) were dissolved in 100 mM phosphate buffer at pH 6.2. Separation of the catechins was carried out on a HPLC with a UV detector, using a Sumichiral HSA column with a mobile phase consisting of 20% ACN with 100 mM phosphate buffer (pH 5.0). The flow rate was 0.9 mL/min, and the elution profiles were monitored by absorbance at 200 nm. The absorption was expressed as the relative absorbance which represents the percentage of full-scale deflection on the recorder expressed from 0 to 100 on the ordinate. This is mentioned in the legend of Fig. 2. (A) EC, (B) EGC, (C) ECg, and (D) EGCg.

Table 1. K_{HSA} and protein binding (%) of cis-type catechins by HPLC with HSA column or ultrafiltration method

Catechins	Retention time (min)	K_{HSA}	Protein binding (%)
EC	2.85 ± 0.04	0.63 ± 0.02[a]	43.7 ± 1.9[a]
EGC	3.19 ± 0.06	0.82 ± 0.03[b]	43.8 ± 1.3[a]
ECg	16.84 ± 0.07	8.62 ± 0.04[c]	96.7 ± 0.6[b]
EGCg	20.87 ± 0.07	10.93 ± 0.04[d]	95.8 ± 1.3[b]

Protein binding (%) were determined by ultrafiltration method. Values are averages with SDs for three experiments. Data were analyzed by Tukey's multiple-range test ($p < 0.01$). Mean values with no letters (a–d) in common are significantly different.

affinity compared to other traditional solution-based methods such as equilibrium dialysis and ultrafiltration method. On the other hand, gallic acid, methyl gallate, and pyrogallol (Fig. 3), which are the model compounds containing a galloyl group or B-ring, weakly bind to HSA (Table 2) and catechins lacking of the galloyl moiety (EC and EGC). These observations indicate that the structural moiety of galloylated catechins (ECg and EGCg), including the basic flavan-3-ol structure, B-ring, and galloyl moiety is insufficient to exert the HSA binding. Furthermore, these findings suggest that not only the combination of the basic flavan-3-ol structure and galloyl group, but also the number of hydroxyl groups on the B-ring has an essential role in the HSA binding ability of tea catechins.

3.2 Binding affinity of *trans*-type catechins for HSA

It has been reported that HSA is able to stereoselectively bind a great number of various endogenous and exogenous compounds. Tea catechins are classified into *cis*-type and *trans*-type based on the configuration of the two hydrogens at the C2 and C3 positions on the C-ring, which has a different configuration from the B-ring (Fig. 4). To examine the steric effects on the interaction of tea catechins with HSA, *trans*-type catechins ((+)-C, (−)-C, Cg, GC, and GCg) were analyzed by HPLC with the immobilized HSA column. The K_{HSA} values obtained from the HPLC analysis are summarized in Table 3. The K_{HSA} values were related as follows: GCg > Cg > > GC ≥ (−)-C and (+)-C. Among the *trans*-type catechins, the maximum value of the K_{HSA} was observed in GCg, and similar to the *cis*-type catechins, the galloylated catechins (Cg and GCg) displayed larger K_{HSA} values than the non-galloylated catechins ((−)-C or GC). In addition, the pyrogallol-type catechins (GC and GCg) displayed larger K_{HSA} values than the catechol-type catechins ((−)-C and Cg). Overall, the K_{HSA} values of *trans*-type catechins ((+)-C, (−)-C, Cg, GC, and GCg) were significantly higher than those of the *cis*-type catechins (EC, ECg, EGC, and EGCg). Although there is a sterical difference between both the C-2 and C-3 positions, no difference in the K_{HSA} values was found between either (−)-C or (+)-C. These results indicate that not only the galloyl moiety, but also the configuration at C-2 and the number of hydroxyl groups on the B-ring are responsible for the binding affinity of tea catechins for HSA.

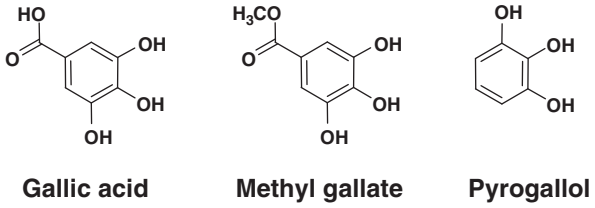


Figure 3. Chemical structures of model compounds containing a galloyl group or B-ring.

Table 2. K_{HSA} of model compounds containing a galloyl group or B-ring by HPLC with HSA column

Catechins	Retention time (min)	K_{HSA}
EGCg	20.87 ± 0.07	10.93 ± 0.04[a]
Gallic acid	3.41 ± 0.06	0.95 ± 0.03[b]
Methyl gallate	2.68 ± 0.03	0.53 ± 0.01[c]
Pyrogallol	2.32 ± 0.04	0.33 ± 0.02[d]

Values are averages with SDs for three experiments. Data were analyzed by Tukey's multiple-range test ($p < 0.01$). Mean values with no letters (a–d) in common are significantly different.

3.3 Binding affinity of methylated catechins for HSA

To examine the influence of the number of hydroxyl groups on the interaction of tea catechins with HSA, methylated catechins were analyzed by HPLC with an immobilized HSA column. Figure 5 shows the structures of the three methylated EGCg derivatives, EGCg-3"OMe, EGCg-4"OMe, and EGCg-4'OMe and their separation on the HSA column. From analyzing the HPLC results, the K_{HSA} values were determined (Table 4). Despite the positional difference of the methyl ether group between the three methylated EGCg derivatives (EGCg-3"OMe, EGCg-4"OMe, and EGCg-4'OMe), the K_{HSA} value of each derivative was considerably lower than that of EGCg itself. These results suggest that the number of hydroxyl groups on the B-ring or the galloyl group plays an important role in the interaction of catechins with HSA and that the binding affinity of tea catechins for HSA is affected by methylation.

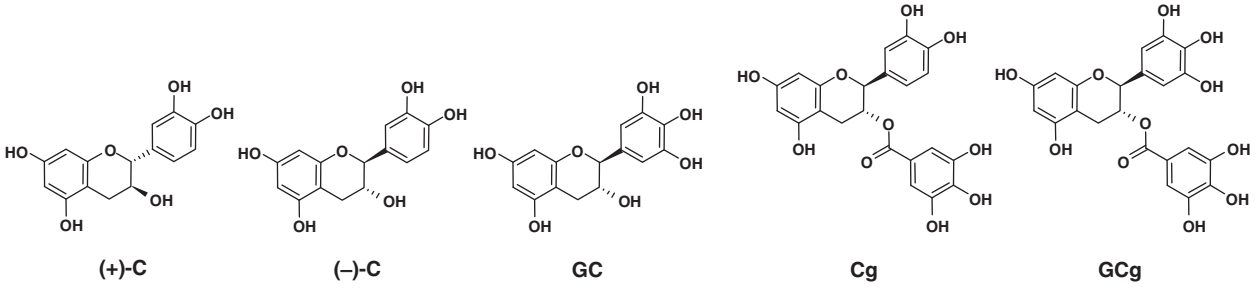
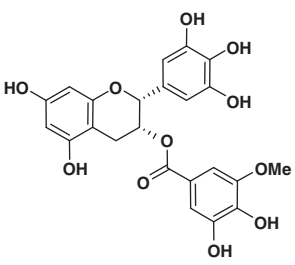
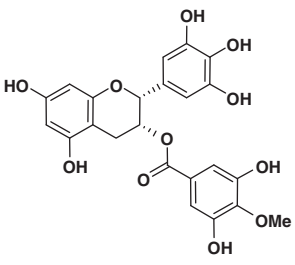


Figure 4. Chemical structures of *trans*-type catechins.



EGCg-3"OMe



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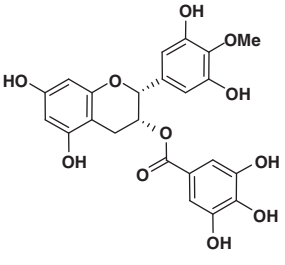

EGCg-4'OMe

Figure 5. Chemical structures of methylated catechins.

Table 3. K_{HSA} of trans-type catechins by HPLC with HSA column

Catechins	Retention time (min)	K_{HSA}
(+)-C	3.02 ± 0.10	0.72 ± 0.06[a]
(−)-C	3.09 ± 0.08	0.77 ± 0.05[ab]
GC	3.31 ± 0.09	0.89 ± 0.05[b]
Cg	18.51 ± 0.09	9.58 ± 0.05[c]
GCg	25.56 ± 0.15	13.60 ± 0.08[d]

Values are averages with SDs for three experiments. Data were analyzed by Tukey's multiple-range test ($p < 0.01$). Mean values with no letters (a–d) in common are significantly different. The values of *trans*-type catechins were significantly higher than those of the *cis*-type catechins in Table 1.

Table 4. K_{HSA} of methylated catechins by HPLC with HSA column

Catechins	Retention time (min)	K_{HSA}
EGCg	20.87 ± 0.07	10.93 ± 0.04[a]
EGCg3"OMe	9.86 ± 0.05	4.63 ± 0.03[b]
EGCg4"OMe	9.76 ± 0.04	4.58 ± 0.02[b]
EGCg4′OMe	11.54 ± 0.03	5.60 ± 0.02[c]

Values are averages with SDs for three experiments. Data were analyzed by Tukey's multiple-range test ($p < 0.01$). Mean values with no letters (a–d) in common are significantly different.

4 Discussion

Identifying target proteins that can interact with food chemicals has been a major focus recently. Searching for the high affinity proteins that bind to food chemicals is the first step in understanding the molecular and biochemical mechanisms behind the functional effects of these chemicals. Recently, methods such as target molecule prediction and affinity chromatography have identified a few proteins that can directly bind with tea catechins, including serum albumin, fibronectin, fatty acid synthase, vimentin, and 67 kDa laminin receptor (67LR) [21–25]. However, as the binding affinity and binding structure have not been determined, the biological and physiological significance of the functional effects of these catechins is not clear. From our evaluation of the binding affinities of catechins and their analogs with HSA, we found that the hydroxyl groups on the galloyl group, the number of hydroxyl groups on the B-ring, and the configuration at C-2 of each catechin influence the affinity for HSA.

Catechins have flavan-3-ol structures with A, B, and C rings, or a galloyl group. The major green tea catechins used in this study included EC, ECg, EGC, and EGCg (Fig. 1) and their C-2 epimeric isomers, (−)-C, Cg, GC, and GCg (Fig. 4). Comparing these nine catechins by HPLC analysis with the HSA column (Tables 1 and 3), we showed that galloylated catechins (ECg, EGCg, Cg, and GCg) have higher binding affinities with HSA than non-galloylated catechins. In addition, pyrogallol-type catechins (EGCg and GCg) have a high affinity compared to catechol-type catechins (ECg and Cg) (Tables 1 and 3). Taken together, an additional insertion of a hydroxyl group at the 5-position in the B-ring appears to contribute to their binding affinities. Furthermore, the binding affinity of *trans*-type catechins (Cg and GCg) was higher than those of the *cis*-type catechins (ECg and EGCg) (Tables 1 and 3). On the other hand, gallic acid, methyl gallate, and pyrogallol, which are the model compounds containing either a galloyl group or B-ring, showed different binding capacities for HSA from catechins lacking of the galloyl moiety including EC and EGC (Tables 1 and 2). These observations suggest that only a triphenol moiety is insufficient to exert HSA binding, and that the combination of the flavan-3-ol structure and galloyl group has an essential role in HSA binding of tea catechins. Moreover, this finding also suggests that the configuration at C-2 as well as the number of hydroxyl groups on the B-ring is also responsible for binding. The most important structural element contributing to HSA binding of tea catechins is the galloyl group, followed by the number of hydroxyl groups on the B-ring or the configuration at C-2. Thus, the galloyl moiety and B-ring attached to flavan-3-ol structure are indispensable for HSA binding of tea catechins.

The importance of the hydroxyl group on the galloyl group and B-ring was confirmed using methylated catechins (EGCg-3"OMe, EGCg-4"OMe, and EGCg-4'OMe) (Fig. 5). HPLC analysis with the HSA column showed that the binding affinities of all the methylated derivatives were lower than that of EGCg (Table 4). Moreover, the

binding affinity of EGCg-4'OMe was shown to be lower than that of ECg (Tables 1 and 4), despite having the same number of hydroxyl groups. These findings suggest that hydroxyl groups on not only the gallate moiety but also on the B-ring are crucial to HSA binding and that binding affinity of tea catechins for HSA is affected by the methyl ether group.

Previous structure–activity analyses of *cis*-type catechins have identified two different substructures that primarily contribute to the biological activities of the catechins. EGCg containing both gallate and pyrogallol moieties exhibited higher activities than pyrogallol moiety-containing EGC or gallate moiety-containing ECg in many reports, suggesting that the gallate and gallyl moiety (B-ring) in the catechins are important for induction of the biological activities [7, 26–28]. Recently, the interaction of EGCg with various proteins and lipids has been proposed to be involved in the biological activity of EGCg. We reported that affinity for lipid bilayers is governed by the presence of the galloyl moiety (*e.g.* ECg > EC; EGCg > EGC), the number of hydroxyl groups on the B-ring (*e.g.* EC > EGC; ECg > EGCg), and the configuration at C-2 (*e.g.* ECg > Cg; EGCg > GCg) [29, 30]. Furthermore, it is also indicated that the Log *P* values of tea catechins in an octanol/water system based on their chemical structure exhibited the hydrophobic property of the catechins. Comparison of Log *P* values (ECg > Cg > EGCg > GCg > > EC > C > EGC > GC) also indicates that the gallate and gallyl moieties (B-ring) in the catechins increase the hydrophobic and hydrophilic properties, respectively, suggesting that the affinity of tea catechins for lipid bilayers is dependent on their lipophilicity. On the other hand, Fujimura *et al.* reported that the number of hydroxyl groups on the B-ring as well as the galloyl moiety is responsible for the cell-surface binding of tea catechins [26]. Comparison of cell-surface binding is as follows: EGCg > GCg > Cg and ECg > > other catechins. The cell-surface receptor 67LR has been identified as crucial factor in the binding of EGCg to the cell surface [25]. The 67LR is also thought to be involved in the cell-surface binding of four galloylated catechins, and the number of hydroxyl groups on the B-ring contributes to 67LR dependency of galloylated catechins on cell-surface binding. Thus, our findings suggest that the number of hydroxyl groups on the B-ring and galloyl moiety also contributes to HSA binding of tea catechins.

This study indicates the galloyl group, the number of hydroxyl groups on the B-ring, and the configuration at C-2 of each catechin influence binding affinity for HSA. The results provide fundamental information on the relationship between the chemical structure of catechins and binding affinity to HSA, and encourage further investigation into the biological activities of tea catechins. Moreover, as EGCg is believed to act as an antioxidant under oxidative stress, its antioxidant activity and the binding capacity of HSA may contribute to the improvement of bioavailability of EGCg as an antioxidant *in vivo*. Our results will provide a basis for future studies of tea catechins and other compounds that can bind to HSA.

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