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Original article

Effect of tea (*Camellia sinensis*) and herbs on advanced glycation endproduct formation and the influence of post-fermentation

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Abstract

***Subjective*:** Tea leaves are rich in polyphenols like catechins which are components that can prevent glycative reactions. This study examined the effect of tea derived from *Camellia sinensis* (CS) and other herb tea on advanced glycation end-product (AGE) formation (anti-glycation activity) and the influence of post-fermentation of tea leaves.

***Methods*:** Sample extracts were prepared from 31 tea leaves (13 CS tea and 18 herb tea), 1 g each of which were incubated in 40 mL hot water at 80°C for 1 hour. Polyphenol samples, 21 kinds, were dissolved in dimethyl sulfoxide. Anti-glycation activity was examined using an *in vitro* albumin/glucose reaction model in which incubation was conducted at 60°C for 40 hours, followed by measurement of AGE-derived fluorescence by a microplate reader and calculation of 50% inhibitory concentration (IC_{50}) of each sample. Total polyphenol content was measured by a Folin-Ciocalteu method and the results were expressed as catechin equivalent (Eq). Aminoguanidine (AG) was used as the positive control.

***Results*:** Anti-glycation activity, the same as or higher than AG, was noted in all the CS tea and in herb tea, rooibos (*Aspalathus linearis*), tien-cha (*Rubus suavissimus*), guava (*Psidium guajava*), and dokudami (*Houttuynia cordata*). In herb tea, the post-fermentation procedure increased the anti-glycation activity and polyphenol content of rooibos, hama-cha (*Chamaecrista nomame*), yien-cha, persimmon (*Diospyros kaki*) and dokudami, while it decreased the activity and polyphenol content in perilla (Shiso-cha; *Perilla frutescens*) and guava. Post-fermentation of CS tea increased the anti-glycation activity and polyphenol content in ishizuchi-kuro-cha, Awa-ban-cha and dan-cha. About half of the 21 polyphenol samples showed anti-glycation activity the same as or higher than AG; it was especially high in quercetagetin and luteolin.

***Conclusion*:** All of the CS-derived tea and some of the herb teas, such as rooibos, tien-cha, guava and dokudami, showed a high anti-glycation activity. In some of the herb teas such as rooibos, hama-cha, yien-cha, persimmon and dokudami, post-fermentation seemed to play a role in polyphenol formation thus increasing the anti-glycation activity.

***KEY WORDS*:** advanced glycation endproducrts (AGEs), tea (*Camellia sinensis*), fermentation, polyphenol, catechin

Introduction

When reducing sugars, *i.e.*, glucose or fructose, and aldehydes bind to protein *in vivo*, the reaction proceeds non-enzymatically and finally forms advanced glycation endproducts (AGEs). This reaction, called glycation, is irreversible and proceeds with aging [1]. AGEs are accumulated in the various tissues and organs, or they bind to specific receptors, thus causing tissue damage and inflammation. Glycative stress is a comprehensive state including stress on the body from the load of reducing sugars and aldehyde and the resulting tissue reactions [2]. Glycative stress is considered to be one of the risk factors which promote age-related deterioration in the body, since glycative stress causes vascular atherosclerosis or bone quality deterioration.

Most teas are made from tea leaves (*Camellia sinensis: CS*) belonging to camellia (Theaceae), of which the hot water extract contains catechins in almost half of the soluble contents. Catechin is a kind of polyphenol (***Fig. 1***) which has a

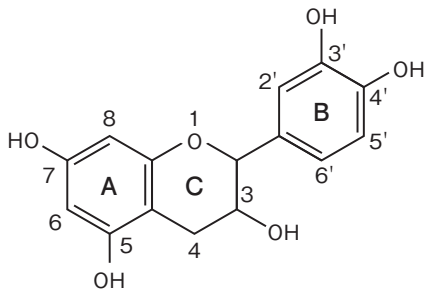

Fig 1. **Polyphenol structure**

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flavonoid structure with a 3-cyclic structure with an A-ring, B-ring and C-ring (***Fig. 2***). There are several kinds of catechins: (±)-catechin (C), a basic form; (-)-epicatechin (EC), a form with sterically different B-ring structure; (-)-epigallocatechin (EGC), a form with hydroxy group combined B-ring; (-)-epigallocatechin gallate (EGCg), EGC combined with gallate group; and (-)-theaflavin, a polymerized product of EC and EGC (***Fig. 3***). Recent research has shown that polyphenols, especially the catechin

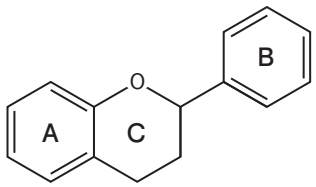

Fig 2. **Flavonoid structure**

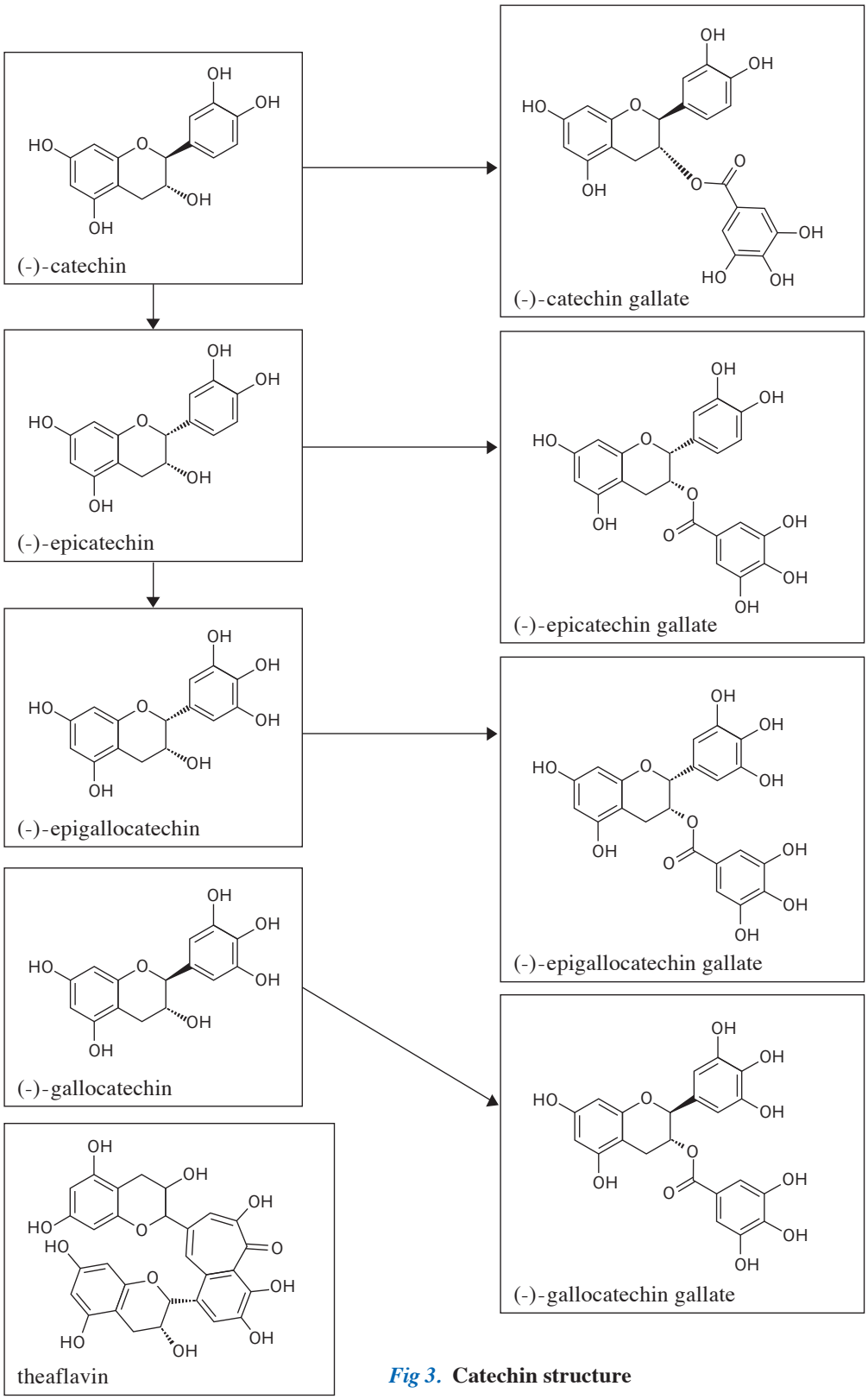

Fig 3. **Catechin structure**

in tea leaves, show anti-glycation activity and there is difference in activity depending on the type of catechins [3,4].

In the present study, the following subjects were used: 13 kinds of CS tea leaves in which catechins are rich, 18 kinds of herb teas in which catechins are less, and 21 kinds of polyphenols in CS tea or herb tea. The anti-glycation activity, AGE generation inhibition, was evaluated in the in vitro reaction between glucose and human serum albumin (HSA), a HSA/glucose model, and the polyphenol amount was measured. Furthermore, the influence of post-fermentation and the relationship between anti-glycation activity and polyphenol structure was examined in the CS tea and herb tea samples.

Methods

Test samples

For the CS tea and herb tea, Darjeeling tea was purchased from Kataoka & Co., Ltd. (Tokyo, Japan), Goishi-cha, Ishizuchi-kuro-cha, Batabata-cha, and Awa-ban-cha were purchased from Furyu (Fukuoka, Japan), and the rest were provided from Hikawa Co., Ltd. (Shimane, Japan). Post-fermentation procedure of the tea leaves was conducted at Hikawa Co., Ltd. For extraction, 1 g of each leaf sample of CS tea and herb tea samples was put into 40 mL of 80°C hot water and incubated at 80°C for 1 hour. Then, 5 mL of each extraction solution was put on an aluminum tray and dried at 120°C for 1 hour; the residue volume was measured and the concentration of the solid content of each sample was calculated. Regarding the polyphenols, chamaemeloside was provided by ARKRAY Inc. (Kyoto, Japan), the rest were obtained from Extrasynthese (Z.I. Lyon, France). Each polyphenol was dissolved in dimethyl sulfoxide (DMSO).

Measurement of inhibitory activity for fluorescent AGE formation

AGE-derived fluorescence was measured as reported previously using a HSA/glucose model [5-7]. Briefly, 100 μL of various concentrations of test sample solutions were added to 500 μL 0.1 mol/L phosphate buffered saline solution (PBS, pH 7.4), 100 μL distilled water, 200 μL 40 mg/mL HSA (Sigma Chemical Co., Ltd., St. Louis, MO, USA), and 100 μL 2.0 mol/L aqueous solution of glucose. Distilled water was then added to make up a total volume of 1.0 mL, and the material was incubated at 60°C for 40 hours (Solution A). Final concentrations were 8 mg/mL HSA and 0.2 mol/L glucose. At the same time, a solution including distilled water added in lieu of aqueous glucose was incubated as a blank for each reaction (Solution B). Solutions prepared without the addition of test samples were incubated as a control (Solution C). And at the same time, a solution including distilled water added in lieu of aqueous glucose was incubated as a blank for controls (Solution D). Fluorescent AGEs were measured quantitatively in each sample reaction solution (A, B, C, and D) to analyze the inhibitory activity for AGE formation. AGE-derived fluorescence was measured using a Spectra Max Paradigm Multi-Mode Detection Platform (Molecular-Devices, CA, USA) microplate reader at an excitation wavelength of 370 nm and a fluorescence wavelength of 440 nm. Calibration curves for inhibition of fluorescent AGE formation (%) were constructed by adding individual samples to a reaction solution at three concentrations (0.1%, 0.01%, 0.001%). The inhibition of AGE formation (%) was calculated using the following formula:

$$\text{Inhibition of fluorescent AGE formation (\%)} = (1-(A-B)/(C-D))\ 100$$

50% inhibitory concentration (IC_{50}) values were then calculated to represent anti-glycation activity. Reported IC_{50} values for AG are 0.063 mg/mL (0.57 mmol/L) [6], 0.068 mg/mL (0.62 mmol/L) [5], and 0.080 mg/mL (0.72 mmol/L) [8]. When the molecular weight of aminoguanidine chloride is 110.5, the anti-glycation activity increases as IC_{50} value reduces.

Polyphenol measurement

The polyphenol content in the samples was measured using the Folin-Ciocalteu (FC) method as previously reported [9-11]. Specifically, 100 μL of each sample solution was added to 50 μL of a 2-fold dilution of FC reagent (Wako Pure Chemical Industries; Chuo-ku, Osaka) and 500 μL of 0.4 M aqueous Na_2CO_3 to make up a total volume of 650 μL; the material was left to stand at ambient temperature for 30 minutes, and absorbency at 660 nm was then measured. A (+)-catechin solution (Wako Pure Chemical Industries) was used as a standard. Using the prepared (+)-catechin calibration curve, the total polyphenol content per solid unit of each vegetable extract solution was calculated as a catechin equivalent (mg catechin Eq/mg solid content). Measurement was repeated three times for each sample.

Results

Anti-glycation activity and total polyphenol content

Anti-glycation activity and total polyphenol content of 31 tea samples are listed in ***Table 1***. Green rooibos (unfermented) was assumed to have no activity because dose-dependent activity was not observed. Of the CS teas, all of the kinds showed anti-glycation activity at the same level or higher than AG, a positive control. Among the herb teas, rooibos (*Aspalathus linearis*), tien-cha (*Rubus suavissimus*), guava (*Psidium guajava*) and dokudami (*Houttuynia cordata*) showed anti-glycation activity at the same level or higher than AG, both before and after post-fermentation.

Of the herb tea samples, there were 5 kinds in which the anti-glycation activity was increased and the relative change of IC_{50} was less than 100% by post-fermentation (relative change values; 21.4% ~ 60.8%): rooibos, Hama-cha (*Chamaecrista nomame*), tien-cha, persimmon (*Diospyros kaki*), and dokudami. Their total polyphenol contents were all increased, with the relative change value being more than 100% (relative change values; 116.0% ~ 274.2%). While, the samples in which the anti-glycation activity was decreased and the relative change of IC_{50} was more than 100 by post-fermentation, were as follows: German chamomile (*Matricaria recutita*) (relative change values; 103.5%), guava (relative change values; 166.4%), and perilla (Shiso-cha; *Perilla frutescens*) (relative change values; 3754.7%). In guava and perilla, the total polyphenol content was increased, and the relative change values were more than 100%. Except for the above 2 samples, the total polyphenol content tended to increase by post-fermentation in samples in which the anti-glycation activity became higher.

CS tea samples, in which anti-glycation activity was

increased and relative change of IC_{50} was less than 100% by post-fermentation, were ishizuchi-kuro-cha, Awa-ban-cha and dan-cha. The relative change values of total polyphenol were 100.0% in ishizuchi-kuro-cha, the same level as that of green tea average, and more than 100% in Awa-ban-cha and dan-cha. Samples, in which relative change of IC_{50} was more than 100% by post-fermentation, were goishi-cha, batabata-cha and pu'er tea. A tendency was not noted in the relative change values of total polyphenols, with 107.0% in goishi-cha, and less than 100% in batabata-cha and pu'er tea. The polyphenol content change by post-fermentation differed according to the specimens.

As for oxidative-fermentation tea, *i.e.* black tea or Oolong tea, in which anti-glycation activity was increased and relative change of IC_{50} was less than 100%, it was only Darjeeling tea in which the relative change value of total polyphenols was more than 100%. The IC_{50} values were increased by oxidative-fermentation in Oolong tea (leaf and tea bag) and black tea. Among them, Oolong tea (tea bag) and black tea showed an increase in total polyphenol content. No relation was noted in the relative change values by oxidative-fermentation between anti-glycation activity and total polyphenol content.

Table 1. Anti-glycation effect (HSA/glucose model) and polyphenol content in CS tea and herb tea

Name	Scientific name	IC_{50} (mg/mL)	% change	Polyphenol (mg catechin Eq/mL)	% change	Character
Herb tea						
Rooibos	*Aspalathus linearis*	0.042		0.827 ± 0.028		OF
+PF		0.009	21.4	1.576 ± 0.007	191.3	OF & PF
Green rooibos	*Aspalathus linearis*	NA		1.830 ± 0.113		
+PF		0.198	–	1.977 ± 0.140	108.0	PF
Hama-cha	*Chamaecrista nomame*	0.255		0.418 ± 0.090		
+PF		0.155	60.8	0.875 ± 0.195	209.4	PF
Tien-cha	*Rubus suavissimus*	0.020		1.495 ± 0.134		
+PF		0.009	45.0	1.731 ± 0.083	116.0	PF
Guava	*Psidium guajava* L.	0.037		2.091 ± 0.176		
+PF		0.062	166.4	0.855 ± 0.168	40.9	PF
Persimon leaf	*Diospyros kaki Thunberg*	0.387		0.254 ± 0.024		
+PF		0.110	28.4	0.697 ± 0.082	274.2	PF
Houttuynia (Dokudami)	*Houttuynia cordata*	0.081		0.658 ± 0.054		
+PF		0.039	48.1	1.565 ± 0.063	237.7	PF
German chamomile	*Matricaria recutita*	0.170		0.204 ± 0.017		
+PF		0.176	103.5	0.239 ± 0.026	117.3	PF
Perilla (Shiso-cha)	*Perilla frutescens var. crispa*	0.159		0.194 ± 0.016		
+PF		5.970	3754.7	0.130 ± 0.017	66.9	PF
Tea						
Green tea (leaf)	CS	0.025		1.481 ± 0.060		
(tea bag)		0.042		1.454 ± 0.267		
Oolong tea (leaf)	CS	0.079	235.3*	1.124 ± 0.149	76.7*	OF
(tea bag)		0.055	163.8*	1.737 ± 0.187	118.4*	OF
Black tea	CS	0.043	128.1*	2.283 ± 0.333	155.6*	OF
Black tea (Darjeeling)	CS	0.022	65.5*	2.245 ± 0.251	153*	OF
Pu'er tea	CS	0.011		2.190 ± 0.305		
+PF		0.017	154.5	1.576 ± 0.058	72	PF
Goishi-cha	CS	0.040	117.7*	1.580 ± 0.096	107*	PF
Ishizuchi-kuro-cha	CS	0.011	32.8*	1.468 ± 0.118	100*	PF
Batabata-cha	CS	0.065	193.6*	0.743 ± 0.011	50.4*	PF
Awa-ban-cha	CS	0.013	38.7*	1.894 ± 0.129	129.1*	PF
Dan-cha	CS	0.024	71.5*	1.976 ± 0.039	133.5*	PF

Data are expressed as mean ± standard error mean. % change, percentage +PF value/value without PF (no mark), percentage sample value/green tea average value of leaf and tea bag (*) and percentage CS, *Camellia sinensis*; NA, no activity; IC_{50}, 50% inhibitory concentration; PF, post-fermentation; OF, oxidative-fermentation.

Anti-glycation activity of polyphenols

To examine the relation between anti-glycation activity and polyphenol structure, the effects of flavonoids which have an apigenin structure (***Fig. 4***) on AGE formation were evaluated. IC_{50} values of 21 polyphenols are presented in ***Table 2***. Among them, 11 of 21 samples showed anti-glycation activity as high as or higher than AG. The activity was highest in quercetagetin (IC_{50}; 0.007 mmol/L, ***Fig. 5***), then luteolin (IC_{50}; 0.034 mmol/L, ***Fig. 6***), while no effect was noted in 7-hydroxyflavone (***Fig. 7***). There was a large difference in anti-glycation activity depending upon the polyphenol type.

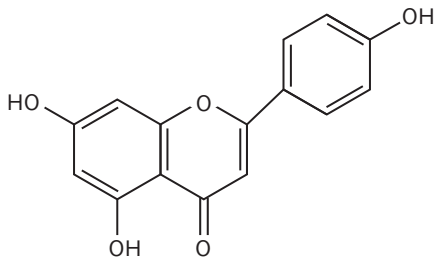

Fig 4. **Structure of apigenin**

Discussion

Anti-glycation activity and polyphenol contents in CS tea and herb tea

The present study showed that anti-glycation activity was high in almost all of the samples of CS tea among 31 kinds of CS tea and herb tea extracts. While, the activity was different depending upon the tea plant type and it was high in rooibos, tien-cha, guava, and dokudami, as high as or higher than AG (IC_{50} the same as or less than AG). The activity was high both before and after post-fermentation in these 4 samples. Regarding the polyphenols, half of 21 samples showed anti-glycation activity as high as or higher than AG, with especially high activity in quercetagetin and luteolin.

After analysis of the influence by post-fermentation on anti-glycation activity and polyphenol contents, a variety of reaction patterns was noted. In rooibos and hama-cha, post-fermentation increased both anti-glycation activity and polyphenol contents, indicating that polyphenols may play a role in anti-glycation activity. While, in German chamomile and guava, post-fermentation decreased the anti-glycation activity but not the polyphenol contents, indicating that factors other than polyphenols may be involved in this reaction. For example, guava is reported to be rich in polyphenols, 90% of which belong to a flavan-type, and most of them are proanthocyanidin polymers [12], however, other components still remain unknown. In ishizuchi-kuro-cha, a post-fermentation product from CS tea, the anti-glycation activity became higher than that before fermentation (*i.e.*, green tea) by the procedure but the polyphenol content was the same level as in green tea. Components other than polyphenols may be involved in the anti-glycation activity of ishizuchi-kuro-cha.

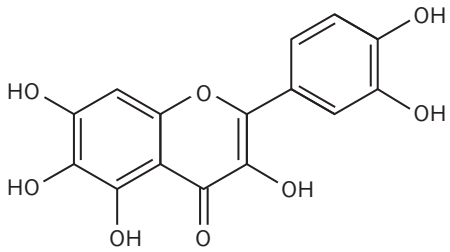

Fig 5. **Structure of quercetagetin**

Polyphenol structure and anti-glycation activity

It is possible that a large part of plant-containing polyphenols may show anti-glycation activity. The experiment using 21 representative flavonoids with an apigenin structure (***Fig. 4***) examined the relationship between anti-glycation activity and flavonoid structure and showed that the activity was wide-ranging depending upon the structure.

In a flavonoid like quercetagetin (***Fig. 5***), the anti-glycation activity was increased when a hydroxy group was added onto the 7th position of the A-ring and, like luteolin (***Fig. 6***), the activity tended to increase when a hydroxy group was added onto the 4th and 5th positions in the B-ring of the flavonoid structure. Like quercetagetin (***Fig. 5***), they tend to lose the activity when a hydroxy group was added onto the 7th position of the A-ring. In contrast, the activity tended to decrease in

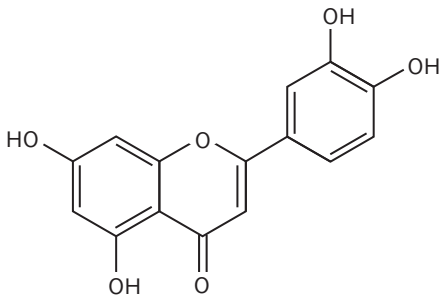

Fig 6. **Structure of luteolin**

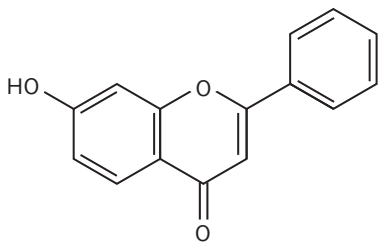

Fig 7. **Structure of 7-hydroxyflavone**

Table 2. Anti-glycation activity of flavonoids

Flavonoid (Polyphenol)	IC_{50} (mmol/L)	hydroxyl moiety position in flavonoid structure		
		A ring	C ring	B ring
Quercetagetin	0.007	5, 6, 7	3	4, 5
Luteolin	0.034	5, 7	N	4, 5
Daidzein [1)]	0.079	7	N	4
Quercetin dehydrate	0.097	5, 7	3	4, 5
Morin	0.127	5, 7	N	4
Chamaemeloside	0.132	5, 7 (methyl-Glu)	N	4
7-Hydroxyflavanone	0.208	7	N	N
Apigenin	0.212	5, 7	N	4
Tricetin	0.225	5, 7	N	3, 4, 5
Liquiritigenin	0.254	7	N	4
Chrysin	0.272	5	N	N
7, 4'-Dihydroxyflavone	0.317	7	N	4
Scutellarein	0.334	5, 6, 7	N	4
Genistein*	0.355	5, 7	N	4
Apigenin-7-O-glucoside	0.357	5, 7 (Glc)	N	4
Naringenin	0.847	7	N	4
4'-Hydroxyflavanone	1.324	N	N	4
Pinocembrin	2.176	5, 7	N	N
5-Hydroxyflavone	4.606	5	N	N
Flavone	1444	N	N	N
7-Hydroxyflavone	31156	7	N	N
AG	0.288	–	–	–

* Isoflavone; IC_{50}, 50% inhibitory concentration; N, none; methyl-Glu, methyl glutamate; Glc, glucoside; AG, aminoguanidine (a positive control).

a flavonoid like naringenin (***Fig. 8***) in which a double bond is deleted between the 2nd and 3rd positions in the C-ring and in an isoflavonoid like liquiritigenin (***Fig. 9***) in which a hydroxy group is deleted from the 5th position in the A-ring.

Conclusion

The present study examined the effect of extracts from 13 CS tea and 18 herb tea samples on AGE formation (anti-glycation activity) using an *in vitro* HSA/glucose reaction model and showed that all of the CS teas and rooibos, tien-cha, guava and dokudami in herb teas have a high anti-glycation activity. In the CS tea and herb tea samples, in which the activity was increased by post-fermentation, also the polyphenols were increased. Since herb tea contains fewer catechins, components other than catechins may play a role in anti-glycation activity. It may be possible to reduce glycative stress by using CS tea or herb tea with high anti-glycation activity combined with our dietary menu in a specific manner.

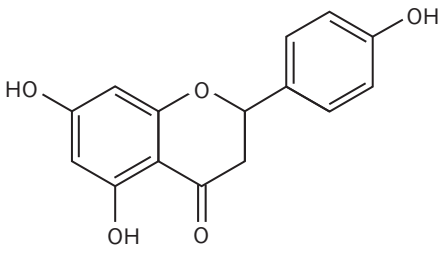

Fig 8. **Structure of naringenin**

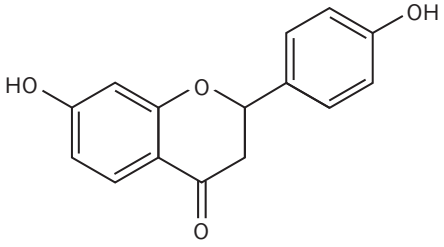

Fig 9. **Structure of liquiritigenin**

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Conflicts of interest statement

The authors have no conflict of interest related to this study to declare.

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