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Structural Requirements of Flavonoids for Inhibition of Protein Glycation and Radical Scavenging Activities

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Abstract—To clarify the structural requirements of flavonoids for formation of advanced glycation end-products (AGEs), various flavonoids were examined. The results suggested the following structural requirements of flavonoids for the inhibition of AGEs formation: (1) as the hydroxyl groups at the 3′-, 4′-, 5-, and 7-positions increased in number, the inhibitory activities became stronger; (2) the activities of flavones were stronger than those of corresponding flavonols, flavanones, and isoflavones; (3) methylation or glucosylation of the 4′-hydroxyl group of flavones, flavonols, and flavanones reduced activity; (4) methylation or glycosylation of the 3-hydroxyl group of flavonols tended to increase activity; (5) glycosylation of the 7-hydroxyl group of flavones and isoflavones reduced activity. In addition, various flavonoids with strong AGEs formation inhibitory activity tended to exhibit strong scavenging activity for 1,1-diphenyl-2-picrylhydrazyl and superoxide anion radicals, with several exceptions.

Introduction

Hyperglycemia, the primary clinical manifestation of diabetes, is associated with development of diabetic complications. Several mechanisms for the development of diabetic complications are reported as follows: (1) increase of polyol pathway flux; (2) increase of advanced glycation end-products (AGEs) formation; (3) activation of protein kinase C isomers; and (4) increase of hexosamine pathway flux.[1] Among them, AGEs are well known to be a cause of aging as well as diabetic complications. As a first step of AGEs formation, proteins in the tissues are modified by reducing sugars (e.g., glucose and fructose). The first step of this modification is the reaction between a free amino group of proteins and a carbonyl group of the sugars, leading to the formation of fructosamines via a Schiff base by Amadori rearrangement. Then important precursors, α-dicarbonyl compounds (e.g., glyoxal, methylglyoxal, and 3-deoxyglucosone), are formed as a result of the initial reaction. Next, the precursors are oxidized and cross-linked, and finally fluorescent AGEs (e.g., pentosidine, and crossline), which are useful markers of AGEs formation, are formed.[2]

Active oxygen species and free radicals react with biomolecular constituents (e.g., lipids, protein, and DNA) to cause certain clinical diseases, such as cerebral ischemia, atherosclerosis, inflammation, diabetes, and cancer.[3] Recently, a hyperglycemia-induced process of overproduction of the superoxide anion radical ($^{\bullet}O_2^-$) by the mitochondrial electron transport chain was reported to partially inhibit the glycolytic enzyme glyceraldehyde phosphatase dehydrogenase, thereby diverting upstream metabolites from glycolysis into the polyol pathway and AGEs formation, etc.[1] In addition, active oxygen species were reported to detect during the glycation processes with traces of metal ions, and strong antioxidants having a phenolic moiety scavenge active oxygen species derived from the glycation processes, and inhibit advanced glycation processes.[4,5]

In the course of our characterization studies on the antidiabetic principles of natural medicines,[6] we previously reported inhibitory activities of 80 flavonoids against aldose reductase, a key enzyme of the polyol pathway, and clarified several structural requirements for the activity.[6g] As a continuing study, inhibitory effects of the flavonoids on bovine serum albumin (BSA) glycation were examined by a fluorescence method. Previously, inhibitory effects of several flavonoids on AGEs formation were reported and their activites in connection with their radical scavenging activities were discussed.[5,7] However, their structure–

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activity relationships and the relation between their inhibitory activities for AGEs formation and their radical scavenging activities were not discussed satisfactorily because of the limited number of compounds. In the present study, 62 flavonoids were examined to clarify further structural requirements of flavonoids for AGEs formation inhibitory activity and the relation between inhibitory activities of flavonoids for AGEs formation and their DPPH radical and $^{\bullet}O_2^-$ scavenging activities.

Results and Discussion

Inhibitory effects of flavonoids on AGEs formation and their structural requirements for the activity

First, to clarify the structure–activity relation of flavonoids for AGEs formation inhibitory activity, inhibitory effects of 62 flavonoids (15 favones, 27 flavonols, 6 flavanoes, 7 isoflavones, 3 flavan-3-ols, and 4 anthocyanins) on AGEs formation were examined using a fluorescent method.[7] As shown in Tables 1–7, 7 flavones [3′,4′-dihydroxyflavone (**5**, IC_{50} = ca. 200 μM), apigenin (**7**, IC_{50} = 172 μM), luteolin (**8,** 99 μM), wogonin (**12**, ca. 200 μM), baicalein (**13**, 93 μM), and luteolins 7-*O*-β-D-glucopyranoside (**14**, 169 μM) and 7-*O*-β-D-glucopyranosiduronic acid (**15**, ca. 200 μM)], 8 flavonols [quercetin (**18**, 151 μM), rhamnetin (**19**, 156 μM), **21** (169 μM), isoquercitrin (**27**, 167 μM), rutin (**28**, 162 μM), rhamnetin 3-*O*-rutinoside (**29**, ca. 200 μM), myricetin (**33**, 119 μM), and myricitrin (**38**, ca. 200 μM)], 3 flavan-3-ols [(+)-catechin (**56**, 112 μM), (−)-epicatechin (**57**, 144 μM), and (−)-epigallocatechin (**58**, ca. 200 μM)], and 4 anthocyanins [cyanidins 3-*O*-β-D-glucopyranoside (**59**, 132 μM) and 3-*O*-rutinoside (**60**, 154 μM) and delphinidins 3-*O*-β-D-glucopyranoside (**61**, 99 μM) and 3-*O*-rutinoside (**62**, 163 μM)] showed substantial inhibitory activities and their activities were stronger than that of a reference compound, aminoguanidine (1.2 mM). On the other hand, a flavone (**1**), flavonols (**24**, **26**, **32**, **34**, **36**, **37**, **42**), flavanones (**43**, **46**, **47**), and isoflavones (**50**, **52**, **53**) lacked the effect (inhibition% at 200 μM: less than 25%), and other flavones (**2**–**4**, **6**, **9**–**11**), flavonols (**16**, **17**, **20**, **22**, **23**, **25**, **30**, **31**, **35**, **39**–**41**), flavanones (**44**, **45**, **48**), and isoflavones (**49**, **51**, **54**, **55**) weakly inhibited the AGEs formation by 25–46% at 200 μM.

By a comparison of the structures of flavonoids with their inhibitory activities, the following structural requirements of flavonoids for the activity were obtained. (1) As the hydroxyl groups of flavones at the 3′-, 4′-, 5-, and 7-positions increased in number, the inhibitory activities became stronger (**1** < **2** < **3** and **4** < **7** < **8**; **6** < **8**). (2) The activities of flavones were stronger than those of corresponding flavonols, flavanones, and isoflavones (**17** < **7**; **18** < **8**; **24** < **11**; **32** < **6**; **48** < **8**; **49** < **4**; **51** < **7**), except for the relation between **1** and **16** (**1** < **16**) and between **9** and **20** (**9** < **20**). Lou et al. reported that methylation or glycosilation of the 3′- or 4′-hydroxyl group reduced the activity from the results of flavonol 3-*O*-glycosides.[5] In agreement with this report, (3) methylation or glucosylation of the 4′-hydroxyl group of flavones, flavonols, and flavanones reduced the activity (**9** < **8**; **20** < **18**; **30** < **29**; **34** < **33**; **46** < **44**; **47** < **45**). (4) Methylation of the 3-hydroxyl group of flavonols tended to increase the activity (**19** ≒ **21**; **22** < **23**; **24** < **25**). (5) Glycosylation of the 7-hydroxyl group of flavones and isoflavones reduced the activity (**14** and **15** < **8**; **50** < **49**; **52** < **51**). (6) The activities of anthocyanins were stronger than those of the corresponding flavonol glycosides (**27** and **28** < **59** and **60**; **38** < **61** and **62**).

DPPH radical and $^{\bullet}O_2^-$ scavenging activities of flavonoids and their structural requirements for the activity

Flavonoids have been recognized largely as beneficial antioxidants that can scavenge harmful active oxygen species including $^{\bullet}O_2^-$, H_2O_2, $^{\bullet}OH$, and $^1O_2$ and there are many reports of structure-antioxidant activity relationships of flavonoids.[8] Although some different results have been reported because of the differences in target active oxygen species or experimental methods, it is generally accepted that the presence of catechol moiety (the 3′,4′-dihydroxyl group) in the B-ring, pyrogallol moiety (the 5,6,7-trihydroxyl group or the 3′,4′,5′-trihydroxyl group) in the A- or B-ring, and the 3-hydroxyl group with the 2,3-double bond in the C-ring are important to scavenge the active oxygen species.

The DPPH radical, which is stable and shows an absorption at 517 nm, has been used as a convenient tool for the radical scavenge assay, and this assay is independent of any enzyme activity.[9] The xanthine–xanthine oxidase system was conventionally used for generation of $^{\bullet}O_2^-$, which was detected by the reduction of nitroblue tetrazolium (NBT) in the present study.[10] Scavenging effects of flavonoids on DPPH radical and $^{\bullet}O_2^-$ have been reported and structure–activity relations were discussed, but experimental protocols and results were different in some cases.[8]

In the present study, 62 flavonoids were examined to clarify the further structural requirements of flavonoids for their radical scavenging activity and relation between inhibitory activities of fravonoids for AGEs formation and their radical scavenging activity. As to DPPH radical scavenging activities, 6 flavones [3′,4′-dihydroxyflavone (**5**, SC_{50} = 6.5 μM), 3′,4′,7-trihydroxyflavone (**6**, 11 μM), luteolin (**8**, 4.8 μM), baicalein (**13**, 13 μM), and luteolins 7-*O*-β-D-glucopyranoside (**14**, 9.2 μM) and 7-*O*-β-D-glucopyranosiduronic acid (**15**, 7.8 μM)], 10 flavonols [kaempferol (**17**, 10 μM), quercetin (**18**, 3.3 μM), **21** (6.0 μM), isoquercitrin (**27**, 4.6 μM), rutin (**28**, 4.3 μM), rhamnetin 3-*O*-rutinoside (**29**, 9.8 μM), fisetin (**32**, 3.7 μM), myricetin (**33**, 4.0 μM), **35** (8.3 μM), myricitrin (**38**, 8.8 μM)], a flavanone [eriodictyol (**48**, 6.5 μM)], 3 flavan-3-ols [(+)-catechin (**56**, 5.9 μM), (−)-epicatechin (**57**, 4.1 μM), and (−)-epigallocatechin (**58**, 2.5 μM)], and 4 anthocyanins [cyanidins 3-*O*-β-D-glucopyranoside (**59**, 3.6 μM) and 3-*O*-rutinoside (**60**, 3.1 μM) and delphinidins 3-*O*-β-D-glucopyranoside (**61**, 3.0 μM) and 3-*O*-rutinoside (**62**, 3.8 μM)] showed substantial DPPH radical scavenging activities with SC_{50} values less than 15 μM and their activities were equivalent to or stronger than that of a reference compound,

α-tocopherol (10 μM). On the other hand, flavones (**1**–**4**, **7**, **9**–**12**), flavonols (**16**, **23**, **25**, **26**, **30**, **31**, **37**, **39**, **41**, **42**), flavanones (**43**–**47**), and isoflavones (**49**–**55**) lacked the effect and other flavonols (**19**, **20**, **22**, **24**, **34**, **36**, **40**) weakly scavenged the DPPH radical with SC_{50} values from 15 to 40 μM (Tables 1–7).

Comparison of the structures of the flavonoids with their inhibitory activities led us to clarify the following structural requirements of flavonoids for the activity. (1) The catechol or pyrogallol moiety at A or B rings in flavones and flavonols was important to show the substantial DPPH radical scavenging activities (**1** < **5**; **3** < **13**; **4** < **6**; **7** < **8**; **17** < **18**), and methylation of the 4′-hydroxyl group of flavones and flavonols tended to reduce the activity (**9** < **8**; **20** < **18**; **22** ≒ **19**; **30** < **29**; **34** < **33**). (2) The activities of flavonols were stronger than those of corresponding flavones (**6** < **32**; **7** < **17**; **8** < **18**; **9** < **20**; **10** < **22**; **11** < **24**), and methylation or glycosylation of the 3-hydroxyl group reduced the activity (**23** < **22**; **25** < **24**; **27** and **28** < **18**; **30** < **22**; **31** < **24**; **37** < **36**; **38** < **33**; **41** < **36**), except for the relation between **19** and **21**, **29** (**19** < **21** and **29**), although Okawa et al. reported that the 3-hydroxyl group of flavonols did not contribute to scavenge for DPPH radicals.[8e] (4) The activities of flavones were stronger than those of flavanones (**48** < **8**). (5) Glycosylation of flavones at the 7-position reduced the activity (**14** and **15** < **8**). (6) Anthocyanins showed stronger activities than the corresponding flavonol glycosides (**27** and **28** < **59** and **60**; **38** < **61** and **62**). In addition, the scavenging activities of anthocyanins with the catechol moiety at the B ring were shown to be equivalent to those of anthocyanins with the pyrogallol moiety (**59** ≒ **61**, **60** ≒ **62**), different from the activities of flavan-3-ols (**57** < **58**).

As to $^{\bullet}O_2^-$ scavenging activities, 7 flavones [3′,4′-dihydroxyflavone (**5**, IC_{50} = 2.5 μM), 3′,4′,7-trihydroxyflavone (**6**, 8.8 μM), luteolin (**8**, 7.8 μM), diosmetin (**9**, 8.0 μM), baicalein (**13**, 10 μM), and luteolins 7-*O*-β-D-glucopyranoside (**14**, 2.2 μM) and 7-*O*-β-D-glucopyranosiduronic acid (**15**, 6.2 μM)], 9 flavonols [kaempferol (**17**, 11 μM), tamarixetin (**20**, 8.0 μM), **21** (8.7 μM), isoquercitrin (**27**, 13 μM), and rutin (**28**, 15 μM), rhamnetin 3-*O*-rutinoside (**29**, 18 μM), **35** (18 μM), myricitrin (**38**, 16 μM), and **39** (11 μM)], a flavanone [eriodictyol (**48**, 7.0 μM)], 3 flavan-3-ols [(+)-catechin (**56**, 5.3 μM), (−)-epicatechin (**57**, 4.1 μM), and (−)-epigallocatechin (**58**, 5.6 μM)], and 2 anthocyanins [delphinidins 3-*O*-β-D-glucopyranoside (**61**, 8.2 μM) and 3-*O*-rutinoside (**62**, 13 μM)] inhibited the NBT formazan formation by $^{\bullet}O_2^-$ less than 20 μM. On the other hand, flavones (**1**, **2**, **4**, **10**–**12**), flavonols (**16**, **23**–**25**, **31**, **36**, **37**, **42**), flavanoes (**43**–**47**), and isoflavones (**49**–**55**) lacked the effects, while flavones (**3**, **7**), flavonols (**19**, **22**, **26**, **30**, **32**, **34**, **40**, **41**), and anthocyanins (**59**, **60**) weakly inhibited the formation of NBT formazan with IC_{50} values from 20 to 100 μM (Tables 1–7). Compounds **5**, **14**, and **20** substantially inhibited the XOD enzyme activity, and compounds **6**, **15**, **21**, **34**, and **48** weakly inhibited the XOD enzyme activity, suggesting that their inhibitions of NBT formazan formation were partially dependent on their XOD enzyme inhibitory activities.

Comparison of their structures with their inhibitory activities indicated the following structural requirements of the flavonoids for such activities. (1) As the hydroxyl groups of flavones at the 3′-, 4′-, 5-, 6-, and 7-positions increased in number, the inhibitory activities tended to become stronger (**2** < **3** < **13**; **6** and **7** < **8**), with some exceptions (**6** < **5**; **7** < **3**). (2) The activities of flavonols were stronger than those of flavones (**7** < **17**). (4) The activities of flavones were stronger than those of isoflavones (**51** < **7**). Anthocyanins with a catechol moiety at the B ring showed weaker activity than the corresponding flavonols glycosides (**59** and **60** < **27** and **28**). In addition, anthocyanins with a pyrogallol moiety at the B ring showed stronger activities than anthocyanins with a catechol moiety (**59** < **61**; **60** < **62**) similar to those of flavonols (**19** < **35**).

Relation between their inhibitory activities for AGEs formation and their scavenging activities for DPPH radical and $^{\bullet}O_2^-$

Lou et al. reported that the AGEs formation inhibitory activities of several flavonoids were in accordance with their DPPH radical scavenging activities.[5] In addition, it was reported that flavonoids might act as metal chelators and/or radical scavengers, and tea catechines such as (−)-epigallocatechin gallate inhibit the formation of AGEs by their radical scavenging activities.[11] In agreement with the previous studies, various flavonoids (**5**, **8**, **13**–**15**, **18**, **21**, **27**–**29**, **33**, **38**, **56**–**62**) with strong AGEs inhibitory activity (IC_{50} = 93–200 μM) tended to show strong scavenging activity for the DPPH radical (IC_{50} = 3.3–13 μM) and/or $^{\bullet}O_2^-$ (IC_{50} = 2.2–18 μM). However, several conflicts were observed; that is, compounds **7**, **12**, and **19** substantially exhibited AGE formation inhibitory activity, but they exhibited weak or less radical scavenging activities, and methylation of the 3-hydroxyl group enhanced the AGEs formation inhibitory activity but reduced DPPH radical scavenging activity. Detailed investigations including in vivo experiments need to be made.

In conclusion, we clarified several structural requirements of flavonoids for AGEs formation inhibitory activity and confirmed the relation of AGEs formation inhibitory activities and scavenging activities for the DPPH radical and $^{\bullet}O_2^-$ in vitro experiments.

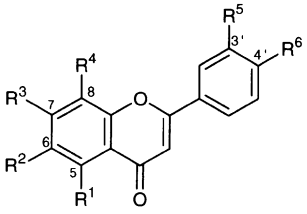
Bioassay Methods

Preparation of flavonoids

Flavonoids **1**–**58** were prepared with some chemical modifications, as described previously.[6g] Anthocyanins **59**–**62** isolated from black currant[12] were kindly provided by Health & Bioscience Laboratories, Meiji Seika Kaisha, Ltd.

Effects on AGEs formation. The AGEs formation was assessed by characteristic fluorescence reported by Morimitsu et al.[7] with slight modifications. Briefly, the reaction mixture of 100 mg D-glucose, 10 mg BSA in 1

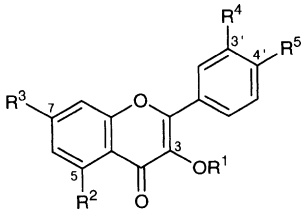
Table 1. Effects of flavones (**1**–**15**) on AGEs formation and their radical scavenging activities



							AGEs Inhibition % at 200 μM	DPPH Radical	$\cdot O_2^-$	
	R^1	R^2	R^3	R^4	R^5	R^6	[IC_{50} (μM)]	SC_{50} (μM)	NBT IC_{50} (μM)	XOD IC_{50} (μM)
Flavone (**1**)	H	H	H	H	H	H	5%	>40	>100	—
7-Hydroxyflavone (**2**)	H	H	OH	H	H	H	27%	>40	>100	—
Chrysin (**3**)	OH	H	OH	H	H	H	43%	>40	20	>30
4′,7-Dihydroxyflavone (**4**)	H	H	OH	H	H	OH	44%	>40	>100	—
3′,4′-Dihydroxyflavone (**5**)	H	H	H	H	OH	OH	49% [ca. 200]	6.5	2.5	7.9
3′,4′,7-Trihydroxyflavone (**6**)	H	H	OH	H	OH	OH	44%	11	8.8	49
Apigenin (**7**)	OH	H	OH	H	H	OH	53% [172]	>40	42	>100
Luteolin (**8**)	OH	H	OH	H	OH	OH	64% [99]	4.8	7.8	>100
Diosmetin (**9**)	OH	H	OH	H	OH	OCH_3	27%	>40	8.0	>100
Pilloin (**10**)	OH	H	OCH_3	H	OH	OCH_3	29%	>40	>100	—
11	OH	H	OCH_3	H	OCH_3	OCH_3	29%	>40	>100	—
Wogonin (**12**)	OH	H	OH	OCH_3	H	H	50% [ca. 200]	>40	>100	—
Baicalein (**13**)	OH	OH	OH	H	H	H	79% [93]	13	10	>30
Luteolin 7-*O*-Glc (**14**)	OH	H	O-Glc	H	OH	OH	53% [169]	9.2	2.2	4.9
Luteolin 7-*O*-GlcA (**15**)	OH	H	O-GlcA	H	OH	OH	51% [ca. 200]	7.8	6.2	29

Glc: β-D-glucopyranosyl; GlcA: β-D-glucopyranosiduronic acid.

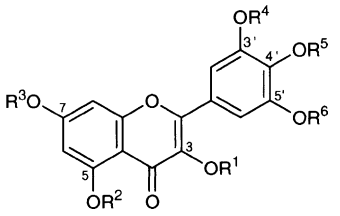
Table 2. Effects of flavonols (**16**–**32**) on AGEs formation and their radical scavenging activities-1



						AGEs Inhibition % at 200 μM	DPPH Radical	$\cdot O_2^-$	
	R^1	R^2	R^3	R^4	R^5	[IC_{50} (μM)]	SC_{50} (μM)	NBT IC_{50} (μM)	XOD IC_{50} (μM)
3-Hydroxyflavone (**16**)	H	H	H	H	H	34%	>40	>100	—
Kaempferol (**17**)	H	OH	OH	H	OH	46%	10	11	>30
Quercetin (**18**)	H	OH	OH	OH	OH	57% [151]	3.3	—	—
Rhamnetin (**19**)	H	OH	OCH_3	OH	OH	55% [156]	21	44	>30
Tamarixetin (**20**)	H	OH	OH	OH	OCH_3	45%	15	8.0	9.4
21	CH_3	OH	OCH_3	OH	OH	57% [169]	6.0	8.7	ca. 30
Ombuine (**22**)	H	OH	OCH_3	OH	OCH_3	27%	18	75	>100
Ayanin (**23**)	CH_3	OH	OCH_3	OH	OCH_3	40%	>40	>100	—
24	H	OH	OCH_3	OCH_3	OCH_3	13%	24	>100	—
25	CH_3	OH	OCH_3	OCH_3	OCH_3	30%	>40	>100	—
26	CH_3	OCH_3	OCH_3	OCH_3	OCH_3	7%	>40	ca. 100	—
Isoquercitrin (**27**)	Glc	OH	OH	OH	OH	52% [167]	4.6	13	>30
Rutin (**28**)	Rut	OH	OH	OH	OH	55% [162]	4.3	15	>100
Rhamnetin 3-*O*-Rut (**29**)	Rut	OH	OCH_3	OH	OH	48% [ca. 200]	9.8	18	>100
Ombuine 3-*O*-Rut (**30**)	Rut	OH	OCH_3	OH	OCH_3	37%	>40	73	>100
31	Rut	OH	OCH_3	OCH_3	OCH_3	39%	>40	>100	—
Fisetin (**32**)	H	H	OH	OH	OH	4%	3.7	80	>100

Glc: β-D-glucopyranosyl; GlcA: β-D-glucopyranosiduronic acid; Gal: β-D-galactopyranosyl; Rha: α-L-rhamnopyranosyl; Rut: α-L-rhamnopyranosyl(1→6)-β-D-glucopyranosyl.

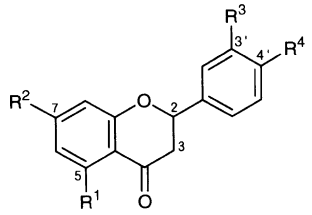
Table 3. Effects of flavonols (**33**–**42**) on AGEs formation and their radical scavenging activities-2



	R^1	R^2	R^3	R^4	R^5	R^6	AGEs Inhibition % at 200 μM [IC_{50} (μM)]	DPPH Radical IC_{50} (μM)	$^{\cdot}O_2^-$ NBT IC_{50} (μM)	$^{\cdot}O_2^-$ XOD IC_{50} (μM)
Myricetin (**33**)	H	H	H	H	H	H	61% [119]	4.0	—	—
Mearnsetin (**34**)	H	H	H	H	CH_3	H	10%	22	21	ca. 100
35	H	H	CH_3	H	H	H	42%	8.3	18	>100
36	H	H	CH_3	CH_3	CH_3	H	17%	39	>100	—
37	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	23%	>40	>100	—
Myricitrin (**38**)	Rha	H	H	H	H	H	52% [ca. 200]	8.8	16	>100
39	Rha	H	CH_3	H	CH_3	H	35%	>40	11	>100
40	Rha	H	H	CH_3	CH_3	H	41%	28	42	>100
41	Rha	H	CH_3	CH_3	CH_3	H	28%	>40	82	>100
42	Rha	H	CH_3	CH_3	CH_3	CH_3	16%	>40	>100	—

Rha: α-L-rhamnopyranosyl.

Table 4. Effects of flavanones (**43**–**48**) on AGEs formation and their radical scavenging activities



	R^1	R^2	R^3	R^4	AGEs Inhibition % at 200 μM [IC_{50} (μM)]	DPPH Radical SC_{50} (μM)	$^{\cdot}O_2^-$ NBT IC_{50} (μM)	$^{\cdot}O_2^-$ XOD IC_{50} (μM)
Flavanone (**43**)	H	H	H	H	9%	>40	>100	—
Liquiritigenin (**44**)	H	OH	H	OH	40%	>40	>100	—
45	H	OCH_3	H	OH	44%	>40	>100	—
Liquiritin (**46**)	H	OH	H	O-Glc	18%	>40	>100	—
47	H	OCH_3	H	O-Glc	18%	>40	>100	—
Eriodictyol (**48**)	OH	OH	OH	OH	46%	6.5	7.0	79

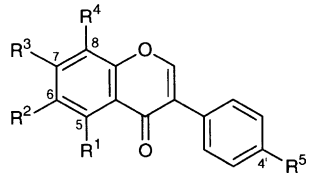
Glc: β-D-glucopyranosyl.

mL sodium phosphate buffer (67 mM, pH 7.2) was incubated at 60 °C for 2 days with or without the test compound. The reaction solution (0.2 mL) was diluted with water (2 mL), and the intensity of fluorescence was measured using a fluorophotometer (Luminescence Spectrometer LS50B, Perkin–Elmer Ltd, Buckinghamshire, England) at an excitation wavelength of 370 nm and an emmision wavelength of 440 nm. The reaction mixture without D-glucose was used as a blank solution. Measurements were performed in duplicate, and the concentration required for a 50% inhibition (IC_{50}) of the intensity of fluorescence was determined graphically. Aminoguanidine was used as a reference compound.

DPPH radical scavenging activity. The free radical scavenging activity of the constituents of flavonoids were assessed using the DPPH radical.[9] An ethanol solution of DPPH (100 μM, 1.0 mL) was mixed with different concentrations of each test compound (0–200 μM, 0.5 mL) and a 0.1 M acetate buffer (pH 5.5, 1.0 mL), and the absorbance change at 517 nm was measured 30 min later. The reaction solution without DPPH was used as a blank test. Measurements were performed in duplicate, and the concentration required for a 50% reduction (50% scavenging concentration, SC_{50}) of 40 μM DPPH radical solution was determined graphically. α-Tocohperol was used as a reference compound.

$^{\cdot}O_2^-$ Scavenging activity. The improved assay method for superoxide dismutase described by Imanari et al. was used.[10] Briefly, a reaction mixture containing 100 μM xanthine, 100 μM EDTA, 25 μM NBT, 0.005% bovine serum albumin, and ca. 1.8 mU/mL xanthine

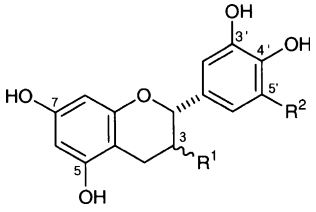
Table 5. Effects of isoflavones (**49**–**55**) on AGEs formation and their radical scavenging activities



	R^1	R^2	R^3	R^4	R^5	AGEs Inhibition % at 200 μM [IC_{50} (μM)]	DPPH Radical SC_{50} (μM)	$^{\bullet}O_2^-$ NBT IC_{50} (μM)	$^{\bullet}O_2^-$ XOD IC_{50} (μM)
Daidzein (**49**)	H	H	OH	H	OH	33%	>40	>100	—
Daidzin (**50**)	H	H	O-Glc	H	OH	−1%	>40	>100	—
Genistein (**51**)	OH	H	OH	H	OH	34%	>40	>100	—
Genistin (**52**)	OH	H	O-Glc	H	OH	12%	>40	>100	—
Tectoridin (**53**)	OH	OCH_3	O-Glc	H	OH	7%	>40	>100	—
Puerarin (**54**)	H	H	OH	Glc	OH	25%	>40	>100	—
Biochanin A (**55**)	OH	H	OH	H	OCH_3	36%	>40	>100	—

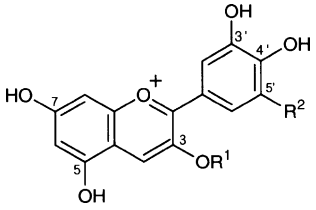
Glc: β-D-glucopyranosyl.

Table 6. Effects of Flavan-3-ols (**56**–**58**) on AGEs formation and their radical scavenging activities



	R^1	R^2	AGEs Inhibition % at 200 μM [IC_{50} (μM)]	DPPH Radical SC_{50} (μM)	$^{\bullet}O_2^-$ NBT IC_{50} (μM)	$^{\bullet}O_2^-$ XOD IC_{50} (μM)
(+)-Catechin (**56**)	β-OH	H	69% [112]	5.9	5.3	>100
(−)-Epicatechin (**57**)	α-OH	H	60% [144]	4.1	4.1	>100
(−)-Epigallocatechin (**58**)	α-OH	OH	48% [ca. 200]	2.5	5.6	>100

Table 7. Effects of anthocyanins (**59**–**62**) on AGEs formation and their radical scavenging activities



	R^1	R^2	AGEs Inhibition % at 200 μM [IC_{50} (μM)]	DPPH Radical SC_{50} (μM)	$^{\bullet}O_2^-$ NBT IC_{50} (μM)	$^{\bullet}O_2^-$ XOD IC_{50} (μM)
Cyanidin 3-*O*-Glc (**59**)	-Glc	H	62% [132]	3.6	57	>100
Cyanidin 3-*O*-Rut (**60**)	-Rut	H	58% [154]	3.1	54	>100
Delphinidin 3-*O*-Glc (**61**)	-Glc	OH	70% [99]	3.0	8.2	>100
Delphinidin 3-*O*-Rut (**62**)	-Rut	OH	56% [163]	3.8	13	>100

Glc: β-D-glucopyranosyl; Rut: α-L-rhamnopyranosyl(→)-β-D-glucopyranosyl.

oxidase in 33.3 mM sodium carbonate buffer (pH 10.2) was incubated with or without each test sample for 20 min at 25 °C (total volume: 3.0 mL). After incubation, the solution was mixed with 0.1 mL of 6 mM $CuCl_2$ to stop the reaction. The formazan formation was monitored at 560 nm. In this assay method, since compounds **18** and **33** reacted with NBT and they showed high optical density in the blank test, the $^{\bullet}O_2^-$ scavenging activities of **18** and **33** could not be determined. In addition, inhibitory effects of test compounds on xanthine oxidase activity were examined to clarify whether the inhibition of formazan formation was due to inhibition of xanthine oxidase. The reaction mixture without NBT was incubated in similar conditions described above and 0.1 mL of 2 M HCl was added to stop the reaction. Uric acid formation was monitored at 290 nm. Several flavonoids (**3**, **13**, **17**, **19**, **21**, **27**) at 100 μM showed high optical density in the blank test, therefore 30 μM was chosen as the maximum concentration. Measurements were performed in duplicate, and the concentration required for a 50% inhibition (IC_{50}) of the NBT formazan formation or uric acid formation was determined graphically.

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