# Chemical Structure-Dependent Differential Effects of Flavonoids on the Catalase Activity as Evaluated by a Chemiluminescent Method

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**The antioxidative activity of flavonoids depends upon a combination of many factors, such as the concentration and chemical structure of the flavonoids and the arrangement of functional groups in their structure. In the present study, to evaluate the antioxidative effect of several types of flavonoids on catalase activity at a physiological $H_2O_2$ concentration, a chemiluminescent (CL) method was used. The $H_2O_2$/luminol-dependent CL intensity in a system containing 3.7 nM catalase and low concentrations (10—100 nM) of green tea flavanols (epigallocatechin gallate; EGCG and epicatechin gallate; EG) was enhanced in comparison with that of a system without catalase, suggesting that EGCG and EG partially suppressed catalase activity. On the other hand, flavone and flavonols such as rutin (a 3-glycosidic flavone), quercitrin (a 3-glycosidic flavonol), myricetin, and kaempferol (flavonols), respectively, lowered the CL intensity to a greater extent at low concentrations (<0.1 $\mu$M) when catalase was present than when catalase was absent, indicating that these flavonoids activate catalase. In addition, isoflavone and flavanone such as daidzein and naringenin, respectively, exhibited weak antioxidative activities against $H_2O_2$ without any effect on the catalase activity over a wide range of flavonoid concentrations (0.04—0.4 $\mu$M). From these results, it was for the first time suggested that the binding of flavonoids to the heme moiety or a protein region of catalase contributes to the enhancement of catalase activity.**

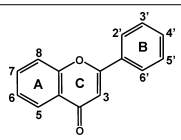
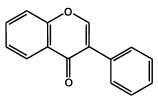
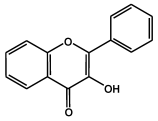
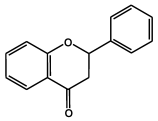
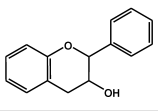
**Key words** flavonoid; antioxidative activity; catalase; chemiluminescence; luminol; reactive oxygen species (ROS) scavenger

Flavonoids are a class of secondary phenolic compounds found in plants and have significant antioxidative and metal-chelating properties. They are widely distributed in nature and mostly concentrated in fruits, vegetables, wines, teas and cocoa. Dietary flavonoids vary not only in the arrangement of their hydroxyl, methoxy, and glycosidic side groups but also in the conjugation between their A- and B-rings. The characteristic features of flavonoids in biological systems are ascribed to their abilities to transfer electrons to free radicals, chelate metal ions,[1] activate antioxidative enzymes,[2] reduce $\alpha$-tocopherol radicals,[3] and inhibit oxidases.[4] Over the past two decades, a considerable amount of data has been accumulated from a number of *in vitro* studies to arrive at a common hierarchy of flavonoids in terms of substitutions and antioxidative activity.

Catalase (EC 1.11.1.6) decomposes hydrogen peroxide ($H_2O_2$) to water and molecular dioxygen and functions as a natural antioxidant by protecting cells against oxidative damage to proteins, lipids and nucleic acids.[5,6] This enzyme has also been used to study the role of reactive oxygen species (ROS) involved in gene expression and apoptosis.[7,8] Although catalase was one of the first enzymes to be isolated and purified, its physiological function and regulation are not yet fully understood.[9] Information regarding the effects of flavonoids on catalase activity is insufficient.

Several techniques based on UV–visible spectrometry,[10] electrochemical detection,[11] and chemiluminescence (CL)[12,13] have been developed to evaluate the interaction of polyphenols with catalase. The UV–visible spectrophotometric assay is most commonly used for determining catalase activity; however, it has some serious disadvantages, as described below. Measurement of $H_2O_2$ at physiological and nontoxic levels, *i.e.*, at concentrations lower than 1 mM, is impossible because this assay has a relatively low sensitivity for $H_2O_2$.[10] In addition, unphysiological high concentrations of substrate $H_2O_2$ in the mM range result in the rapid inactivation of catalase.[14,15] Furthermore, molecular dioxygen liberated in a gaseous form during the enzyme reaction leads to disturbance of the absorbance.[14] The CL method is thus considered to be the most sensitive because it does not require an excitation light source as in the case of fluorometric and spectrophotometric analyses. Ogawa *et al.*[12] reported the successful application of luminol CL for detecting the antioxidative activities of catechin. In this study, to evaluate the antioxidative effect of different types of flavonoids (Table 1)

Table 1. Classification and Structure of Flavonoids Used in This Study

| Class | General structures | Flavonoids (substitutions) |
|---|---|---|
| Flavone |  | Rutin (5,7,3′,4′-OH; 3-rutinose) |
| Isoflavone |  | Daidzein (7,4′-OH) |
| Flavonol |  | Quercitrin (5,7,3′,4′-OH; 3-rhamnose)<br>Myricetin (3,5,7,3′,4′,5′-OH)<br>Kaempferol (3,5,7,4′-OH) |
| Flavanone |  | Naringenin (5,7,4′-OH) |
| Flavanol |  | Epicatechin gallate (EG) (5,7,3′4′-OH; 3-gallate)<br>Epigallocatechin gallate (EGCG) (5,7,3′,4′,5′-OH; 3-gallate) |

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on catalase activity at physiological $H_2O_2$ concentrations, the $H_2O_2$/luminol-dependent CL method was used. The results revealed that flavonoids exhibit different modes of enhancement on catalase activity depending on their structure.

## MATERIALS AND METHODS

**Reagents** Catalase from bovine liver, epicatechin gallate (EG), ruthin hydrate, quercitrin, myricetin, kaempherol, naringenin, and daidzein were purchased from Sigma (St. Louis, MO, U.S.A.). Hydrogen peroxide ($H_2O_2$), sodium hypochlorite (NaClO), potassium dihydrogenphosphate, and epigallocatechin gallate (EGCG) were from Wako Pure Chemicals (Tokyo, Japan). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and dimethyl sulfoxide (DMSO) were purchased from Dojindo (Kumamoto, Japan). Luminol and disodium hydrogen phosphate were from Nacalai Tesque (Kyoto, Japan). 2-Methyl-6-phenyl-3,7-dihydroimidazo-[1,2-a]-pyrazin-3-one (*cypridina hilgendorfii* luciferin analog; CLA) was purchased from Tokyo Kasei Organic Chemicals (Tokyo, Japan). Distilled water was purified using a Millipore ultrafiltration unit (Tokyo, Japan).

**Solutions** A stock solution of 2 mM luminol was freshly prepared in 20 mM Na/K phosphate buffer (pH 7.4). A stock solution of 0.2 mM CLA was freshly prepared in 20 mM Na/K phosphate buffer (pH 7.4). Stock solutions of NaClO and $H_2O_2$ dissolved in the Na/K phosphate buffer were freshly prepared daily. Their concentrations were determined spectrophotometrically.[16] Stock solutions of 10 mM each flavonoid dissolved in DMSO were also prepared daily prior to use.

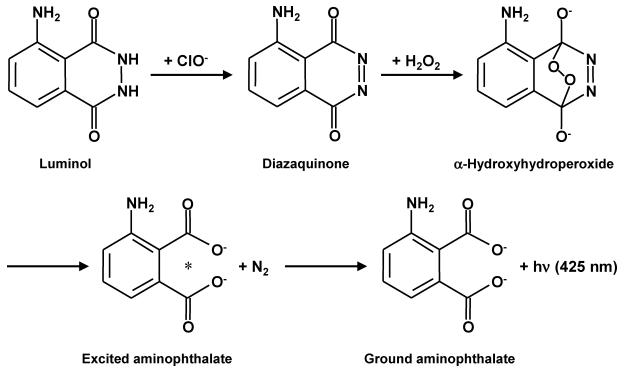
**Measurements of Chemiluminescence** CL emission light was measured by an Aloka Model BLR-201 luminescense reader (Tokyo, Japan) at 30 °C. The sample system (total volume=1.25 ml) consisting of 0.85 ml of 5.5 nM catalase (final concentration: 3.7 nM) dissolved in 20 mM Na/K phosphate buffer (pH 7.4) and 0.1 ml of 0.1—5 $\mu$M of each flavonoid (final concentration: 0.008—0.4 $\mu$M) was preincubated at 30 °C for 10 min. Subsequently, 0.1 ml of 440 $\mu$M DTPA (final concentration: 35 $\mu$M) and 0.05 ml of 50 $\mu$M luminol (final concentration: 2 $\mu$M) were added to the control (without catalase) and sample systems. After adding 0.1 ml of 550 $\mu$M $H_2O_2$ (final concentration: 44 $\mu$M), the reaction was initiated by rapid injection of 0.05 ml of 30 $\mu$M NaClO (final concentration: 1.2 $\mu$M) as the oxidant for luminol (from luminol to diazaquinone),[13,17,18] and the CL intensity was simultaneously measured and recorded for 1 min. The control system without catalase was also subjected to CL measurements under the same conditions.[13,17,18]

In order to evaluate specifically the generation of singlet oxygen ($^1O_2$) through the reaction of $H_2O_2$ and $ClO^-$, 0.05 ml of 25 $\mu$M CLA (final concentration: 1 $\mu$M) were added to the control (without catalase) and sample systems, as substitute for luminol in the system. It is known that CL is observed during the reaction of $^1O_2$ with CLA.[17,18] After adding $H_2O_2$ (final concentration: 44 $\mu$M), the reaction was initiated by rapid injection of NaClO (final concentration: 1.2 $\mu$M), and the generated CL intensity was simultaneously measured and recorded for 1 min.

**Statistical Analysis** The CL intensity due to the reaction of flavonoids with a low concentration of $H_2O_2$ in the absence or presence of catalase was determined and compared using the Student's or unpaired *t* test. Experimental results are expressed as the means±standard deviations of 4—6 repeated measurements.

## RESULTS AND DISCUSSION

It is known that CL occurs as a result of a chemical reaction with some form of ROS. Luminol is oxidized by HClO ($ClO^-$) to diazaquinone in a two-electron oxidation and further specifically converted by $H_2O_2$ to an excited aminophthalate *via* an $\alpha$-hydroxyhydroperoxide,[19] the reaction scheme of which is described as follows.[20,21]




Previous investigations have shown that the small luminescence signal generated in this reaction is linear when the concentration of $H_2O_2$ is in the nM to $\mu$M range.[13,22] In our study, the CL assay has been used as an indispensable analytical method for determining $H_2O_2$ and evaluating the antioxidative activity of flavonoids.[18] The present method has several advantages due to its rapidity, sensitivity, reproducibility, and simplicity.

Addition of NaClO to a mixture of luminol, DTPA, and $H_2O_2$ resulted in the development of high luminescence that was dependent on the $H_2O_2$ concentration.[13] No CL was detected after the addition of NaClO when catalase was incubated with luminol and DTPA in the absence of $H_2O_2$ (data not shown). The $H_2O_2$-dependent CL emission is indeed controlled by two-electron oxidation of the luminol/NaClO system[13,18—22] and is not influenced by other mechanisms such as those involving catalase and other peroxidases.[19,23]

Because the myeloperoxidase (MPO)-mediated generation of $^1O_2$ was suggested in the MPO/$H_2O_2$ system through the generation of HClO by MPO,[24,25] the generation of $^1O_2$ was evaluated in our system, using CLA in place of luminol. Addition of NaClO to a mixture of CLA, DTPA, and $H_2O_2$ resulted in the weak luminescence less than 20 kilocpm (data not shown), indicating that $^1O_2$ is a minor species in this system. Although the potent generation of $^1O_2$ was observed in the mixture of higher concentration of NaClO (0.5—25 mM) and $H_2O_2$ (5—100 mM).[18,26] the level of $^1O_2$ chemically generated in our system seems to be very low due to the reaction of $\mu$M level of NaClO and $H_2O_2$. Then, the luminol-dependent chemiluminescence is considered to be due to the reaction of $H_2O_2$ with luminol oxidized by $ClO^-$.[13,18—22]

After the addition of $H_2O_2$ and NaClO to the mixture of catalase, DTPA, and luminol, high CL intensity was observed with increasing concentrations of $H_2O_2$ as shown in

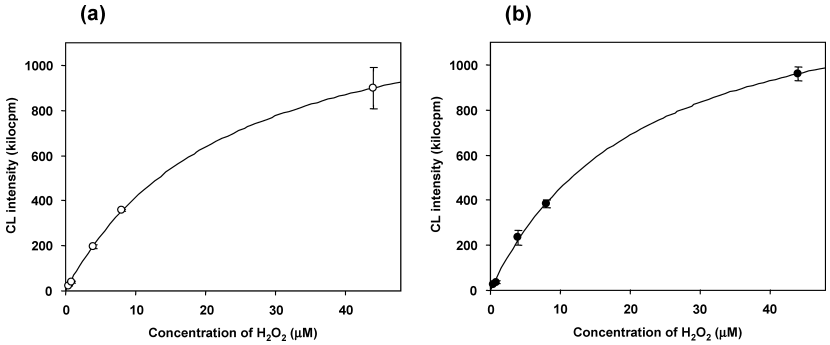



Fig. 1. Profiles of $H_2O_2$/Luminol-Dependent CL Intensity *vs.* $H_2O_2$ Concentration (a) without Catalase and (b) with 3.7 nM Catalase

The system contained the same concentrations of reagents, and the concentration of $H_2O_2$ was changed from 0.4 to 44 $\mu$M. Each symbol shows the mean values±standard deviations for 4 repeated measurements. The theoretical curves were derived from calculations with a non-linear least-squares regression method based on a Michaelis–Menten type of equation ($CL=CL_{max}\cdot C/(K_m+C)$, $C=H_2O_2$ concentration). The theoretical curves fitted well with the observed CL profiles with and without catalase. The values of $CL_{max}$ and $K_m$ were estimated to be 1370±20 kilocpm and 23±1, respectively, in the control system (without catalase), and 1410±40 kilocpm and 22±1, respectively, in the sample system (with 3.7 nM catalase), indicating no significant difference between the systems with and without catalase in terms of the $H_2O_2$-dependent CL emission profiles.

Fig. 1 where the Michaelis–Menten type of equation ($CL=CL_{max}\cdot C/(K_m+C)$, $C=H_2O_2$ concentration) was fitted well with the observed CL profiles in the samples with and without catalase. Kinetic parameters in Fig. 1 such as the $CL_{max}$ (maximum CL intensity) and $K_m$ of the $H_2O_2$ concentration were estimated to be 1370±20 kilocpm and 23±1 $\mu$M, respectively, in the control system (without catalase) and 1410±40 kilocpm and 22±1 $\mu$M, respectively, in the sample system (with 3.7 nM catalase). This indicated that there was no significant difference between the systems with and without catalase in terms of the $H_2O_2$-dependent CL emission profiles, and that the native activity of 3.7 nM catalase to scavenge $H_2O_2$ was ignored in the absence of flavonoids.

Previous studies had demonstrated the strong antioxidative effects of green tea polyphenols, including catechins such as epigallocatechin gallate (EGCG) and epicatechin gallate (EG).[27,28] On the other hand, apoptosis-associated characteristics in human oral tumor cell lines were induced more efficiently by EGCG than by ascorbates,[29] gallic acid,[5] vitamin K,[30] flavonoids[31] or steroidal saponins.[32] Since catalase partially inhibited the cytotoxic activity of EGCG, the possible involvement of $H_2O_2$ in cell death induction was investigated using bis(2,4,6-trichlorophenyl)oxalate (TCPO) CL method.[33] Under optimal conditions, 1 mM EGCG has been reported to produce 1 mM $H_2O_2$.[33]

In our experiments, we used lower concentrations of flavonoids and catalase, and unexpectedly found that the CL intensity in the system with catalase and flavanols such as EGCG and EG was enhanced in comparison with that in the system without catalase (Figs. 2a, b). The fact that $H_2O_2$ production by EGCG and EG at low concentrations (final concentration: 8—80 nM) was partially promoted by the addition of catalase suggested that these flavanols partially suppressed the catalase activity. These results are consistent with those of the peroxalate CL experiment.[33] Previous results from ESR spectroscopy demonstrated that EGCG and EG produced their organic radicals under alkaline conditions, however, the radical intensity completely disappeared in the samples containing catalase, suggesting the possible interaction of EGCG and EG with catalase.

In studies on the interaction of antioxidants such as rutin (a 3-glycosidic flavone) with rutinose, the additional iron-chelating site does not make any remarkable contribution to the antioxidant activity.[27,34] The glycoside provides a chelation site; however, this site is not expected to play an important role when other chelation sites are available.[27] Rutin was shown to have no pro-oxidant activities either in the presence or absence of iron.[35] In our experiments, rutin decreased the CL intensity in the control and sample systems to a considerable extent (Fig. 2c). Slight activation of catalase was observed only with 0.064 $\mu$M rutin. Whether or not the glycosylation of the flavonol increases its antioxidative activity against $H_2O_2$ was next examined by a procedure similar to that used in the case of the flavone. Quercitrin (a 3-glycosidic flavonol) also exhibited a higher antioxidant activity against $H_2O_2$ with slight activation of catalase (Fig. 2d).

Myricetin (flavonol) has been found to be a potent inhibitor of glutathione reductase (GR) and catalase.[2] Hydroxyl groups in the B-ring of flavonoids are important for enzyme inhibition. Myricetin posseses a pyrogallol moiety in the B-ring; thus, this compound is used to form superoxide anions *via* the reduction of molecular dioxygen under conditions in which stable free radicals are formed. Therefore, compounds with pyrogallol moieties exhibit a pro-oxidant property that in turn counteracts their antioxidative effects.[34] Our data showed that the CL intensity in a system containing myricetin and catalase was lower at low concentrations of myricetin when catalase was present than when catalase was absent, indicating that myricetin activates catalase *in vitro* at low concentrations, *i.e.*, less than 0.1 $\mu$M (Fig. 2e). In flavonoids containing a single OH group in the B-ring, the rest moiety of the flavonoid structure appears to become more important for the ROS scavenging activity than in the case of the catechol flavonoids. Kaempferol (flavonol) is known to be an extremely active ROS scavenger, and its activity lies in the range of that of the catechol flavonoids.[34] We found that kaempferol in a concentration range less than 0.1 $\mu$M is a more potent activator of catalase than myricetin (Fig. 2f). The glycosylation of flavonoids was reported to reduce their activity in comparison with the corresponding aglycones.[27] However, blocking the 3-OH group with rhamnose in the C-ring of quercitrin resulted in a higher antioxidant activity against $H_2O_2$ with slight activation of catalase (Fig. 2d).

The effect of daidzein (isoflavone) on the antioxidative enzymes in rat hepatoma H411E cells was studied by Rohrdanz

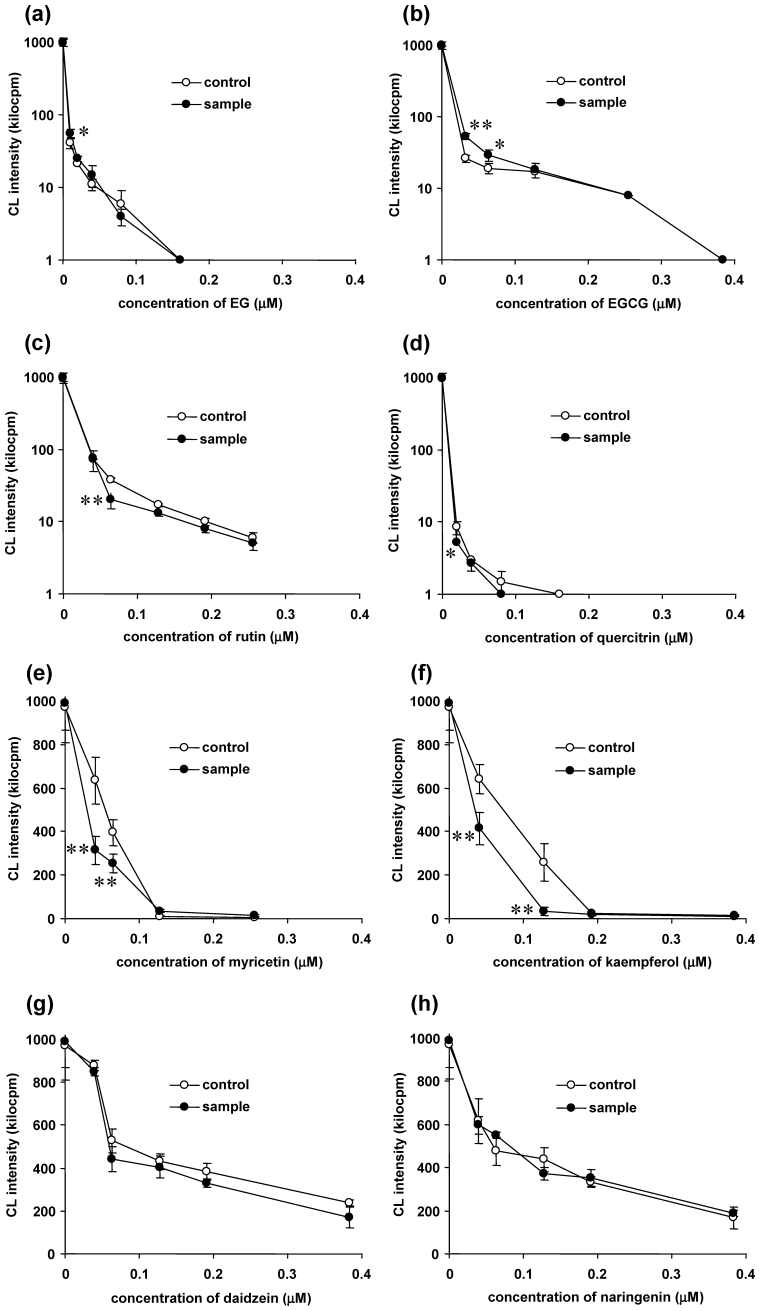



Fig. 2. Effects of Flavonoids with Different Structures on Catalase Activity

The sample system containing 0.008—0.4 $\mu$M flavonoid and 3.7 nM catalase was incubated at 30 °C for 10 min. First, 35 $\mu$M DTPA and 2 $\mu$M luminol were added, followed by 44 $\mu$M $H_2O_2$; this was followed by rapid injection of 1.2 $\mu$M NaClO. The CL intensity was measured for 1 min. A control system without catalase was also prepared, and measurements were carried out in the same manner as those for the sample system. The CL intensity was detected as the peak height value (kilocpm) at each flavonoid concentration. The *y*-axis in (a)—(d) is in the semi-logarithmic scale, while the *y*-axis of other figures is in the normal scale. Each symbol shows the mean values±standard deviations of 4—6 repeated measurements. Significant difference: * $p<0.05$ and ** $p<0.01$ *vs.* control system.

*et al.*[36] Daidzein itself exerted a mild oxidative stress.[36] In our experiment, daidzein was shown to have weak antioxidative activity against $H_2O_2$ without any effect on catalase (Fig. 2g).

Flavonones with a single OH group in the B-ring (naringenin) have been suggested to possess little antioxidative activity in lipid system. The glycosylation of the 7-OH group in a structure with a saturated heterocyclic ring and a single OH group on the B-ring strongly suppresses the antioxidative activity.[27] In the case of naringenin, we also observed weak antioxidative activity without any effect on the catalase activity over a wide range of flavonoid concentrations (0.04—0.4 $\mu$M) (Fig. 2h).

This study demonstrated that the catalase activity increases depending on the structure of antioxidative flavonoids and their derivatives, as summarized in Table 2. A combination of many factors such as the position of functional groups on the flavonoid structure and their interaction with catalase, including that of the ferriprotoporphyrin group, also contribute to this effect. In particular, the substitutions at C-3 in flavonoids are regarded to be important in terms of both the antioxidative potency against $H_2O_2$[37] and

Table 2. Relationship among Antioxidative Activity, Structure, and Effect on Catalase Activity of Flavonoids Used in This Study

| Compounds | Apparent $IC_{50}$ value (nM) for scavenging $H_2O_2$ | Class | C-3 | OH substitution | Catalase activity |
|---|---|---|---|---|---|
| EGCG | 16.4±4.4 | Flavanol | Gallate | 5,7,3′,4′,5′ | Suppression |
| EG | 5.4±0.8 | Flavanol | Gallate | 5,7,3′,4′ | Suppression |
| Rutin | 21.6±6.8 | Glycoside flavone | Rutinose | 5,7,3′,4′ | Enhancement |
| Quercitrin | 10.1±2.0 | Glycoside flavonol | Rhamnose | 5,7,3′,4′ | Enhancement |
| Myricetin | 53.8±8.6 | Flavonol | OH | 5,7,3′,4′,5′ | Enhancement |
| Kaempferol | 75.6±9.8 | Flavonol | OH | 5,7,4′ | Enhancement |
| Naringenin | 62.2±8.1 | Flavanone | H | 5,7,4′ | No effect |
| Daidzein | 90.4±8.8 | Isoflavone | Phenyl | 7,4′ | No effect |

$IC_{50}$ value was obtained from the relationship between CL intensity and concentration of flavonoids as shown in Fig. 2.

the enhancing effect on catalase activity. However, we don't find out at the present stage the clear relationship between the apparent $IC_{50}$ values for scavenging $H_2O_2$ and the enhancing effects on catalase activity.

Although there is data on the importance of antioxidants, the relationship between the antioxidant activity and chemical structure of flavonoids is not yet fully examined. Analysis of the complicated mechanism of activation or the inhibition profiles of flavonoids in catalase will be difficult. From these results, it was for the first time suggested that the binding of flavonoids to the heme moiety or a protein region of catalase contributes to the enhancement of catalase activity. In the next stage, studies on the binding of flavonoids to catalase, such as those on the interaction of these compounds with the heme group or protein moieties of catalase will be necessary.

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