Relationships between Structure and Anti-oxidative Effects of Hydroxyflavones

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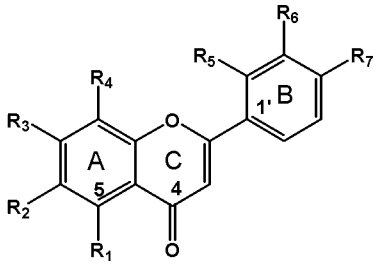
Primuletin, chrysin, and luteolin show vasorelaxing, anti-oxidative, and chemopreventive effects, respectively.[1-3] All of these molecules are flavones, which are subclasses of flavonoids with a common feature of a C6-C3-C6 skeleton. Flavonoids can be classified on the basis of the substitution patterns of the C ring, such as flavanone, flavonol, isoflavone, and flavone. Among these, flavone, flavanone, and flavonol are determined by the C3 ring in the C6-C3-C6 skeleton, which is named the C ring. Flavones contain both a ketone group at the C-4 position and a double bond between C-2 and C-3 of the C ring. Note that primuletin, chrysin, and luteolin have only minor differences in their hydroxyl groups, but show considerable differences in their biological activities. To elucidate the relationships between the structures of flavones and their biological activities based on the numbers and/or positions of hydroxyl groups, we examined the anti-oxidative effects of 20 hydroxyflavone derivatives. The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay was used to screen for anti-oxidative effects in our study.

Twenty hydroxyflavones consisting of five monohydroxyflavones, nine dihydroxyflavones, four trihydroxyflavones,

Table 1. Nomenclature and structures of hydroxyflavone derivatives and their scavenging effects.



Derivative	Nomenclature	R_1	R_2	R_3	R_4	R_5	R_6	R_7	Scavenging effects (%)
1	Flavone	H	H	H	H	H	H	H	0.0
2	5-Hydroxyflavone (Primuletin)	OH	H	H	H	H	H	H	0.2
3	6-Hydroxyflavone	H	OH	H	H	H	H	H	7.9
4	2'-Hydroxyflavone	H	H	H	H	OH	H	H	3.8
5	3'-Hydroxyflavone	H	H	H	H	H	OH	H	5.9
6	4'-Hydroxyflavone	H	H	H	H	H	H	OH	4.2
7	5,7-Dihydroxyflavone (Chrysin)	OH	H	OH	H	H	H	H	5.4
8	5,3'-Dihydroxyflavone	OH	H	H	H	H	OH	H	2.0
9	5,4'-Dihydroxyflavone	OH	H	H	H	H	H	OH	5.9
10	7,2'-Dihydroxyflavone	H	H	OH	H	OH	H	H	3.1
11	7,3'-Dihydroxyflavone	H	H	OH	H	H	OH	H	5.5
12	7,4'-Dihydroxyflavone	H	H	OH	H	H	H	OH	4.0
13	2',3'-Dihydroxyflavone	H	H	H	H	OH	OH	H	87.8
14	2',4'-Dihydroxyflavone	H	H	H	H	OH	H	OH	40.4
15	3',4'-Dihydroxyflavone	H	H	H	H	H	OH	OH	84.0
16	5,7,2'-Trihydroxyflavone	OH	H	OH	H	OH	H	H	9.7
17	5,3',4'-Trihydroxyflavone	OH	H	H	H	H	OH	OH	81.0
18	6,7,3'-Trihydroxyflavone	H	OH	OH	H	H	OH	H	87.8
19	7,8,4'-Trihydroxyflavone	H	H	OH	OH	H	H	OH	87.7
20	5,7,3',4'-Tetrahydroxyflavone (Luteolin)	OH	H	OH	H	H	OH	OH	84.8

one tetrahydroxyflavone, and one flavone were purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ) and used without any further purification due to their purities of approximately 98%. Their nomenclatures and structures are listed in Table 1. DPPH radical-scavenging effects were tested according to the method of Park *et al.*[4] The scavenging effects of the 20 hydroxyflavones are listed in Table 1.

To determine the relationships between the structures of these compounds and their anti-oxidative effects, we performed a three-dimensional (3D) quantitative structure-activity relationship (QSAR) study using a comparative molecular field analysis (CoMFA).[5] In the absence of any information regarding the biological target, indirect ligand-based approaches such as 3D-QSAR can assist in clarifying the SARs. All calculations were carried out using the molecular modeling package SYBYL v7.2 (Tripos Inc., St. Louis, MO) running on Red Hat Enterprise Linux workstations. All chemical structures of hydroxyflavone derivatives were built utilizing the SYBYL/Sketch module and minimized by a conjugate gradient method using the Tripos force field of SYBYL.[6] The minimum energy discrepancy of 0.001 kcal/mol was set as a convergence criterion. Data sets were randomly separated into training and test sets composed of 16 and 4 compounds, respectively. The training set was used to create QSAR models and the test set was used to validate the models. The log values were applied as experimental data for the DPPH assay due to the large differences in percent values among the compounds. Alignment using a template was performed to overlay the molecules approximately in the same configuration.[7] 6,7,3'-Trihydroxyflavone (**18**) was chosen as a template and the remaining derivatives were aligned to it using the Align Database method of SYBYL. The aligned derivatives are shown in Fig. 1.

CoMFA was carried out to evaluate the steric and electrostatic properties associated with the activities of the derivatives. The partial least-squares analysis method was used to linearly correlate the CoMFA fields to biological activity. Cross-validation was performed using the leave-one-out method in which one compound was removed from the data set and its activity was predicted using the model derived from the remaining molecules in the data set. Equal weights for CoMFA were assigned to steric and electrostatic fields using the CoMFA standard scaling option. The statistical correlations were examined to evaluate the predictive ability of the generated models. The cross-validated q^2 and conventional correlation coefficient r^2 were calculated based on the overall compounds in the training set.

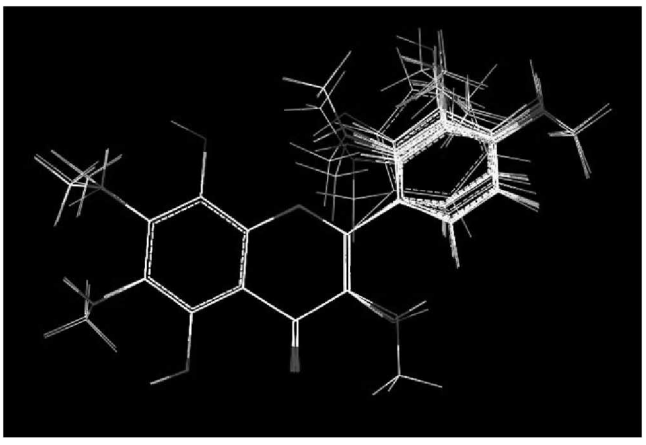


Figure 1. Alignment of the training set, with 6,7,3'-trihydroxyflavone used as a template.

Vitamin C was used as a reference and its scavenging effect was 89.1% in our experiment. The results obtained from DPPH radical-scavenging experiments indicated that all flavonoids examined in this study do not have anti-oxidative effects. Specifically, the scavenging effects of monohydroxyflavones ranged from 0% to 10%. In the case of dihydroxyflavones and trihydroxyflavones, some compounds were found to compete with vitamin C, while others showed scavenging effects below 10%. 3D-QSAR was performed to examine the relationship between the structure and scavenging effect. Of the 20 compounds tested in this study, 16 were selected for the training set, and the remaining four compounds, 5,7-dihydroxyflavone (**7**), 7,2'-dihydroxyflavone (**10**), 2',3'-dihydroxyflavone (**13**), and 3',4'-dihydroxyflavone (**15**), were used as the test set for QSAR. Compounds used as the training set were superimposed based on the A and C rings of the flavone backbone (Fig. 1).

Results obtained from the partial least-squares analysis showed a cross-validated q^2 value of 0.56 using the leave-one-out method, indicating a correlation probability of less than 5%. The conventional correlation coefficient r^2 value was 0.96. Experimental values of scavenging effects and their predicted values obtained from QSAR are listed in Table 2. A plot on a log scale of the experimental to predicted values is shown in Fig. 2.

Here, the residual values between the experimental and predicted values ranged within 0.2, with the exception of 5,3'-dihydroxyflavone. The predicted values of four derivatives used, **7**, **10**, **13**, and **15**, as a test set were 4.9%, 5.6%, 58.9%,

Table 2. The log scales of the experimental and predicted values for the training set and the residuals.

Derivatives	Experimental data	Predicted values	Residuals
1	0.00	0.02	−0.02
2	0.00	−0.19	0.19
3	0.90	0.99	−0.09
4	0.58	0.68	−0.10
5	0.77	0.57	0.20
6	0.62	0.63	−0.01
8	0.31	0.69	−0.38
9	0.77	0.76	0.01
11	0.74	0.69	0.01
12	0.61	0.75	−0.14
14	1.61	1.55	0.06
16	0.99	0.89	0.10
17	1.91	1.77	0.14
18	1.94	1.91	0.03
19	1.94	1.94	0.00
20	1.93	1.96	−0.03

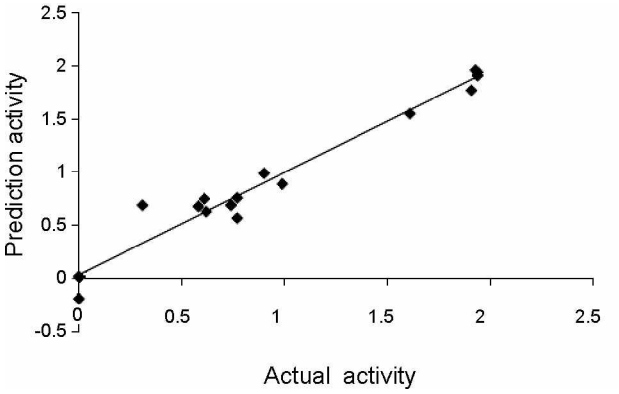


Figure 2. Plot of the experimental values *vs.* their predicted values of scavenging effects for the training set.

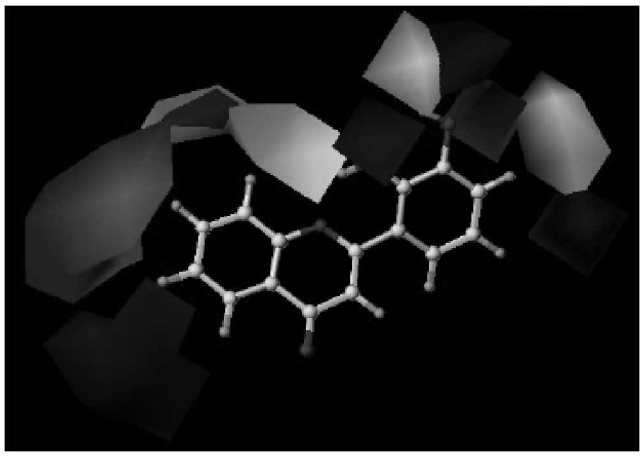


Figure 3. CoMFA steric and electrostatic contour maps of 2',3'-dihydroxyflavone showing the best scavenging effect.

and 87.1%, respectively, and their respective experimental values were 5.4%, 3.1%, 87.8%, and 84.0%. The contour maps obtained from CoMFA indicated that the 3D-QSAR method is useful for recognizing structure-activity relationships. The red contours, representing electronegative properties, favor anti-oxidative effects and the blue contours indicate the reverse. The yellow contours favor steric bulky substituents. As shown in Fig. 3, two red contours were located at 6-OH, 7-OH, and 8-OH of the A ring, and at 3'-OH and 4'-OH of the B ring.

Among the 16 training sets, the scavenging effects of 6,7,3'-trihydroxyflavone, 7,8,4'-trihydroxyflavone, 5,3',4'-trihydroxyflavone, and 5,7,3',4'-tetrahydroxyflavone were over 80%. In addition, three yellow contours were placed at 2'-OH, 3'-OH, 4'-OH, and 8-OH as shown in Fig. 3. This high scavenging rate was satisfied with 5,3',4'-trihydroxyflavone and 5,7,3',4'-tetrahydroxyflavone. In the case of 5,7,2'-trihydroxyflavone, despite the presence of 7-OH and 2'-OH, its effect was only 9.7%. Similarly, 7,4'-dihydroxyflavone showed only a 4.0% scavenging effect. Therefore, it was concluded that hydroxyl groups should be placed at the *ortho* position. These observations demonstrated that separated hydroxyl groups do not contribute to the anti-oxidative effect. Thus, the scavenging effects of 2',3'-dihydroxyflavone and 3',4'-dihydroxyflavone were 87.8% and 84.0%, respectively, while that of 7,2'-dihydroxyflavone was 3.1%. These results were confirmed in four test sets.

Park *et al.*[4] reported that flavones substituted with 3- and/or 6-hydroxyl groups and 7- and/or 4'-methoxyl group show good scavenging effects. In addition to this report, Young *et al.*[8] showed that 3-, 2'-, and/or 4'- hydroxyl groups result in high scavenging effects. Although no 3-OH substituted flavones were tested in this study, our previously published results[4,8] agreed well with the observation that 6-OH, 2'-OH, and 4'-OH are required for good anti-oxidative effects. 5,7,3',4'-Tetrahydroxyflavone (luteolin) induces the activity of superoxide dismutase, which can repair cells damaged by free radicals in the body.[9] Thus, luteolin has a good anti-oxidative effect and our results accordingly indicated a scavenging effect of 84.8%. In contrast, 5,7-dihydroxyflavone (chrysin) exhibits weak superoxide scavenging activity, as reported previously,[10] and its anti-oxidative effect in the present study was 5.4%. Although luteolin and chrysin belong to the flavones, they show very different anti-oxidative effects. The results of the present study indicate that these differences are due to the substitution of their hydroxyl groups.

Experimental0 Section

To test the DPPH radical-scavenging effects, 100 μL aliquots of each sample were adjusted to 0.1% methanol solution.[11-12] Then, 1 mL of 100 mM Tris-HCl buffer (pH 7.4) was added, and the mixture was added to 1 mL of a 0.5 mM methanol solution of DPPH radicals. After 15 min at 37 °C, absorbance at 517 nm was measured with a spectrophotometer (Shimadzu, Tokyo, Japan). The scavenging effects were calculated using the following equation[4]: scavenging effects (%) = [1-(absorbance of sample/absorbance of control)] × 100.

Initially, the steric and electrostatic CoMFA potential fields were calculated at each lattice intersection having a regularly space grid of 2.0 Å. The grid box dimensions were then determined automatically and the region boundaries were enlarged over 4 Å in each direction from the coordinates of each derivative. The van der Waals potential and Coulombic terms representing steric and electrostatic fields, respectively, were calculated by the standard Tripos force fields using an Sp^3 carbon probe with the van der Waals radius of 1.52 Å and +1 charge, respectively. The steric and electrostatic energies were truncated to +30 kcal/mol and the electrostatic contribution was ignored at lattice points with maximal steric interactions.

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