

## Full Paper

**Kaempferol Derivatives Prevent Oxidative Stress–Induced Cell Death in a DJ-1–Dependent Manner**Wei Qu<sup>1,2</sup>, Li Fan<sup>3</sup>, Yun-chul Kim<sup>1</sup>, Shizuma Ishikawa<sup>4</sup>, Sanae M.M. Iguchi-Ariga<sup>4</sup>, Xiao-Ping Pu<sup>2,\*a</sup>, and Hiroyoshi Ariga<sup>1,\*b</sup><sup>1</sup>Laboratory of Molecular Biology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan<sup>2</sup>Department of Molecular and Cellular Pharmacology, <sup>3</sup>Department of Natural Medicines, School of Pharmaceutical Science, Peking University Health Science Center, Beijing 100191, P.R. China<sup>4</sup>Laboratory of Environmental Molecular Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

Received February 12, 2009; Accepted April 19, 2009

**Abstract.** DJ-1, a causative gene product of a familial form of Parkinson's disease (PD), PARK7, plays a role in anti-oxidative stress, and loss of its function is thought to result in the onset of PD. Superfluous oxidation of cysteine at amino acid 106 (C106) of DJ-1 renders DJ-1 inactive, and such oxidized DJ-1 was observed in patients with the sporadic form of PD. In this study, we examined the relationship between DJ-1 and compounds extracted from traditional Chinese medicines possessing anti-oxidant activity. Of the 12 compounds tested, 5 were found to specifically bind to the C106 region by using a quartz crystal microbalance. Although 4 compounds prevented rat PC12 and primary neuronal cells from undergoing H<sub>2</sub>O<sub>2</sub>-induced cell death, the protective activity of 2 compounds, kaempferol 3-*O*- $\beta$ -rutinoside and 6-hydroxy-kaempferol 3,6-di-*O*- $\beta$ -D-glucoside, was diminished in cells transfected with siRNA targeting DJ-1, indicating DJ-1–dependent reaction of these compounds. Furthermore, these compounds reduced the level of reactive oxygen species and restored tyrosine hydroxylase activity that had been induced and compromised, respectively, by treatment of cells with H<sub>2</sub>O<sub>2</sub>. The results suggest that these compounds are useful lead compounds for PD therapy.

**Keywords:** DJ-1, traditional Chinese medicine, cell death, oxidative stress, Parkinson's disease

**Introduction**

Traditional Chinese medicines (TCM) have long been used for treatment of many diseases, including inflammation, ischemic stroke, and cancer (for reviews, see references. 1–3). TCM have been used as complementary or alternative medicines to supplement or replace treatments prescribed by licensed medical practitioners or as sources of compounds that would be modified as so-called “western medicine”.

Parkinson's disease (PD) is caused by dopaminergic cell death, and genetic and environmental factors are

thought to affect the onset of PD. These factors lead to oxidative stress, mitochondrial dysfunction, and impairment of the protein degradation system, resulting in cell death. Although a precursor of dopamine and inhibitors of dopamine degradation have been used for PD therapy, cell death progresses during treatment. Identification of compounds or proteins that inhibit oxidative stress–induced neuronal cell death is necessary. DJ-1 was first identified by our group as a novel oncogene product (4) and later found to be a causative gene product of a familial form of PD, PARK7 (5). DJ-1 plays roles in transcriptional regulation (6–8) and the anti-oxidative stress reaction (9–12), and loss of its function is thought to result in the onset of PD. DJ-1 has three cysteines at amino acid numbers 46, 53, and 106 (C46, C53, and C106, respectively). Of the three cysteine residues, oxidation of C106 is necessary for

\*Corresponding authors.

\*<sup>a</sup>puxp2020@gmail.com \*<sup>b</sup>hiro@pharm.hokudai.ac.jp

Published online in J-STAGE on June 5, 2009 (in advance)

doi: 10.1254/jphs.09045FP

DJ-1 to exert its activity (11 – 13). Further oxidation of C106 is, on the other hand, thought to render DJ-1 inactive (14), and such oxidized DJ-1 has been observed in patients with the sporadic form of PD and Alzheimer's disease (15, 16). We have shown that administration of DJ-1 protein dramatically prevented dopaminergic cell death and restored the locomotion defect in PD model rats into which 6-hydroxydopamine had been injected, suggesting that DJ-1 is a pharmaceutical target for PD (17). Likewise, intrastriatal injection of DJ-1 protected against neurodegeneration caused by focal cerebral ischemia and reperfusion in rats (18). Furthermore, we

have identified small molecular weight compounds that bind to the C106 region of DJ-1, and these compounds, like DJ-1 protein, prevented dopaminergic cell death and restored the locomotion defect in PD and stroke model rats (19, 20). These compounds were found to maintain an active form of DJ-1 by preventing excess oxidation of DJ-1 (19).

In this study, we screened DJ-1-binding compounds extracted from TCM and found that some compounds prevented oxidative stress-induced death of neuronal cells in a DJ-1-dependent manner.

**Table 1.** Origins of compounds and their activities

Name	Compound	Origin of plant	Effect	Reference
hhw1	hydroxysafflor yellow A	<i>Carthamus tinctorius L.</i>	Anti-oxidant Anti-platelet aggregation Neuroprotective Cardioprotective Anti-inflammation Protects against cerebral ischemic injury	33 – 35
hhw5	6-hydroxykaempferol 3,6,7-tri- <i>O</i> - $\beta$ -D-glucoside	<i>Carthamus tinctorius L.</i>	Anti-oxidant Anti-platelet aggregation	
hhw10	6-hydroxykaempferol 3- <i>O</i> - $\beta$ -D-glucoside	<i>Carthamus tinctorius L.</i>	Anti-oxidant Anti-platelet aggregation	
hhw11	6-hydroxykaempferol 3- <i>O</i> - $\beta$ -D-rutinoside	<i>Carthamus tinctorius L.</i>	Anti-oxidant Anti-platelet aggregation	
hhw12	kaempferol 3- <i>O</i> - $\beta$ -D-sophoroside	<i>Carthamus tinctorius L.</i>	Anti-oxidant Anti-platelet aggregation	
hhw14	kaempferol 3- <i>O</i> - $\beta$ -D-rutinoside	<i>Carthamus tinctorius L.</i>	Anti-oxidant Anti-platelet aggregation	
hhw16	anhydrosafflor yellow B	<i>Carthamus tinctorius L.</i>	Anti-oxidant Anti-platelet aggregation Neuroprotective effects against brain ischemic injury	36
hhw19	6-hydroxykaempferol 3,6-di- <i>O</i> - $\beta$ -D-glucoside	<i>Carthamus tinctorius L.</i>	Anti-oxidant Anti-platelet aggregation	
hhw20	6-hydroxykaempferol 3- <i>O</i> - $\beta$ -rutinoside-6- <i>O</i> - $\beta$ -D-glucoside	<i>Carthamus tinctorius L.</i>	Anti-oxidant Anti-platelet aggregation	
hhw21	(2 <i>S</i> )-4',5-dihydroxyl-6,7-di- <i>O</i> - $\beta$ -D-glucopyranosyl flavanone	<i>Carthamus tinctorius L.</i>	Anti-oxidant Anti-platelet aggregation	
hhw29	quercetin 3- <i>O</i> - $\beta$ -D-rutinoside	<i>Carthamus tinctorius L.</i>	Anti-oxidant Anti-platelet aggregation Anti-bacterial activity Anti-tumor Anti-inflammatory effect	37
	quercetin	<i>Carthamus tinctorius L.</i>	Anti-oxidant Anti-platelet aggregation Anti-cancer Anti-obesity Anti-diabetes	27, 28

## Materials and Methods

### Compounds

The compounds used in this study are shown in Table 1. The hhw series of compounds and quercetin were extracted from *Carthamus tinctorius L.*

### Cell culture and knockdown of DJ-1 expression

Rat PC12 cells were cultured in Dulbecco's modified Eagle's medium with 5% calf serum and 10% horse serum. The nucleotide sequences of the upper and lower strands of oligonucleotides of siRNA targeting the rat *DJ-1* gene are 5'-GGCUUUGGAUGCAAGGUU ATT-3' and 5'-UAACCUUGCAUCCAAAGCCTT-3', respectively. siRNA and control siRNA targeting the *DJ-1* and luciferase gene, respectively, were transfected into PC12 cells by lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

### Western blotting

To examine the expression levels of proteins in cells, proteins were extracted from cells with a buffer containing 150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0), and 0.5% NP-40. Proteins were then separated on a 12.5% polyacrylamide gel containing SDS and subjected to western blotting with the respective antibodies. Proteins on the membrane were reacted with an IRDye800 (Rockland, Gilbertsville, PA, USA) or Alexa680-conjugated second antibody (Molecular Probe/Invitrogen) and visualized by using an infrared imaging system (Odyssey; LI-COR, Lincoln, NE, USA). The antibodies used were anti-actin (1:4,000; Chemicon, Temecula, CA, USA) and anti-DJ-1 (1:2,000) antibodies. The rabbit anti-DJ-1 antibody was prepared by using the peptide corresponding to amino acid numbers 179–189 of human DJ-1 as an immunogen.

### Cell viability assay

Cells were cultured in a 96-well plate and treated with various amounts of hydrogen peroxide. Cell viability was then measured by an MTT assay using a cell counting kit-8 (DOJINDO, Kumamoto).

### Binding of compounds to the C106 region by a quartz crystal microbalance (QCM)

Fixation of compounds on a sensor chip of QCM (Affinix Q, Initium, Tokyo) was carried out as follows. The sensor chip was washed with a solution containing H<sub>2</sub>O<sub>2</sub> and sulfonic acid (molar ratio = 1:3), and then it was incubated with 4  $\mu$ l of 1  $\mu$ M compound dissolved in chloroform until the solution had evaporated. To the sensor chips fixed with compounds in Affinix Q, 8  $\mu$ l of

1 mg/ml wild-type and C106S DJ-1 and bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) was applied, and their frequency was measured according to the manufacturer's protocol.

### Primary neuronal culture of the ventral mesencephalon

Cultures of the rat mesencephalon were established according to methods described previously (21). The ventral two-thirds of the mesencephalon were dissected from rat embryos on the 17th–19th days of gestation. The dissected regions included dopaminergic neurons from the substantia nigra and the ventral tegmental area but not noradrenergic neurons from the locus ceruleus. Neurons were dissociated mechanically and plated out onto 0.1% polyethyleneimine-coated, 24-well plates at a density of  $2.5 \times 10^6$  cells/well. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum for 2 days and DMEM containing 2% B-27 supplement (Invitrogen) and 2 mg/ml aphidicolin (Sigma-Aldrich) without fetal calf serum from the 3rd day onwards. The animals were treated in accordance with guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

To examine the effects of DJ-1-binding compounds on oxidative stress-induced cell death, the cells were cultured in the presence or absence of 1  $\mu$ M each compound for 10 min and then treated with 150–250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h. Cell viabilities were then examined by an MTT assay.

### Detection of production of ROS

PC12 cells were pretreated with 1  $\mu$ M of each compound for 10 min and then treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h. The cells were stained with DAPI and incubated with 5  $\mu$ M DCFH-DA for 10 min at 37°C and visualized under a scanning microscope (Bioevo BZ-9000; Keyence, Osaka).

### Activity of tyrosine hydroxylase

PC12 cells were treated with 1  $\mu$ M of each compound for 10 min and 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h. Tyrosine hydroxylase (TH) activity was measured according to the published method using HPLC (8). The HPLC column used was a 4.6  $\times$  150 mm COSMOSIL column (Nacalai Tesque, Kyoto).

### Statistical evaluation

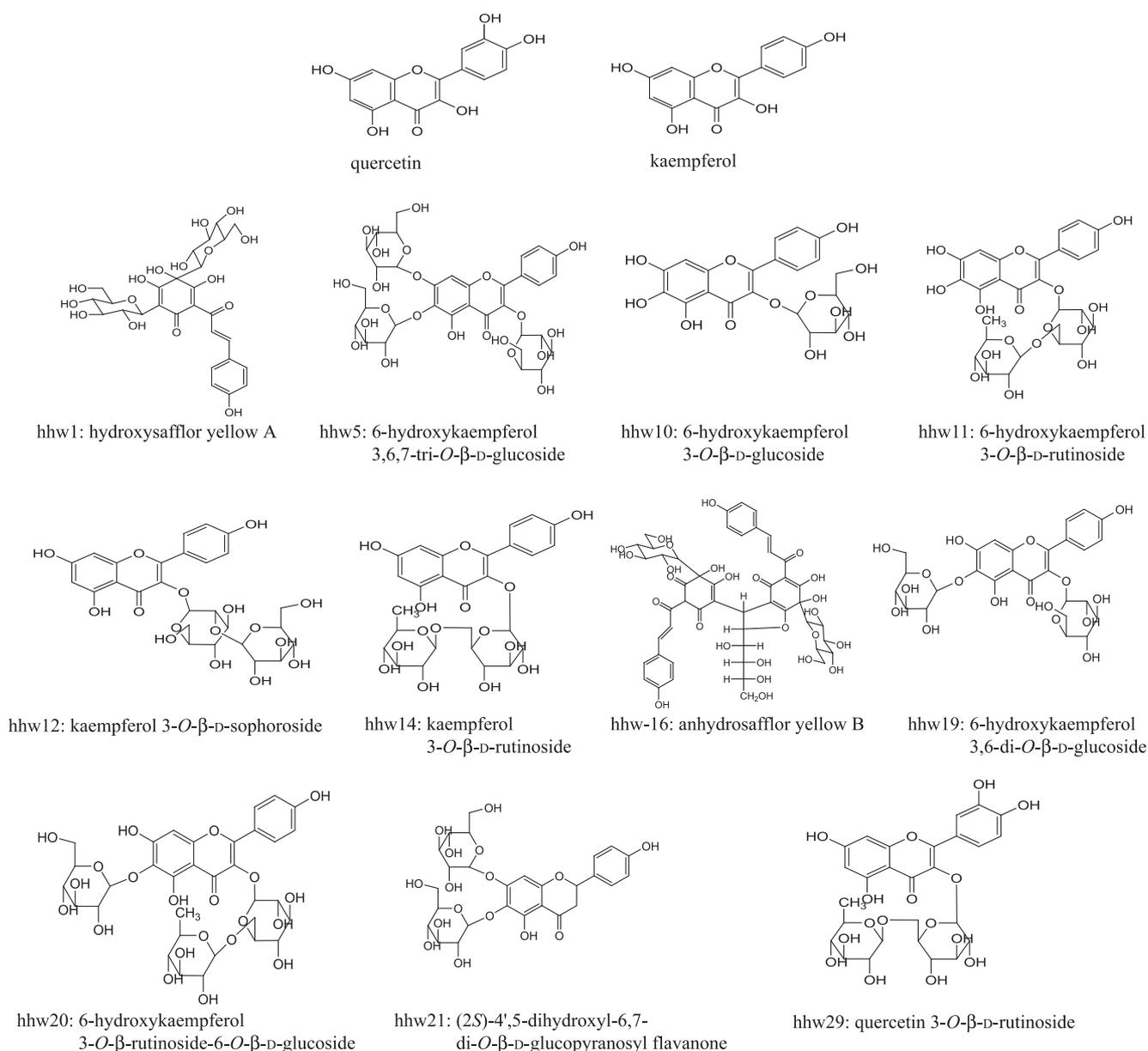
All data are shown as the mean  $\pm$  S.E.M. The significance of differences was determined by one-way or repeated measures analysis of variance (ANOVA). Further statistical analysis for *post hoc* comparisons was performed using the Bonferroni/Dunn test (StatView; Abacus Concepts, Berkeley, CA, USA).

## Results

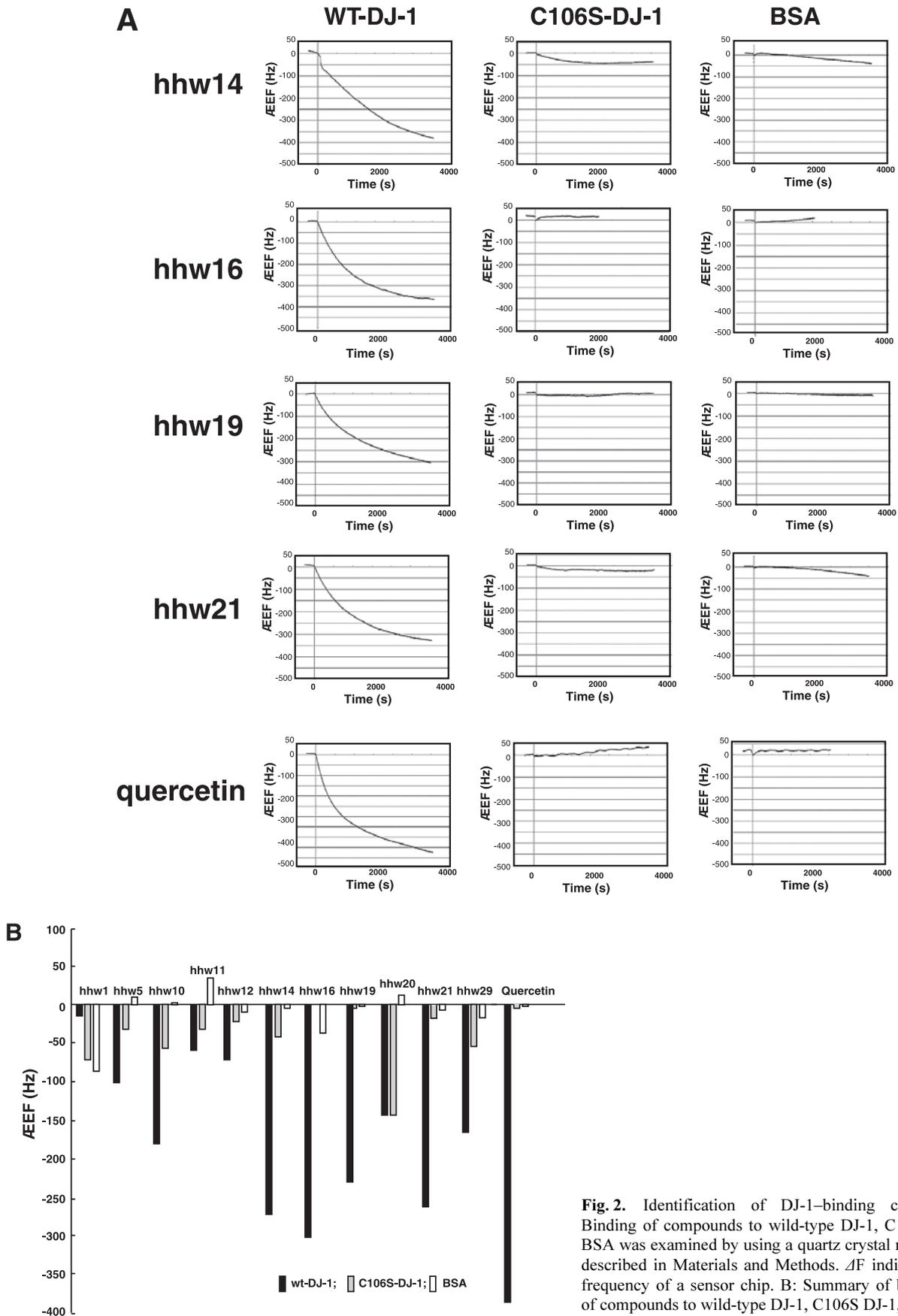
### Identification of DJ-1-binding compounds

We examined 13 derived from TCM: hydroxysafflor yellow A (hhw1), 6-hydroxykaempferol 3,6,7-tri-*O*- $\beta$ -D-glucoside (hhw5), 6-hydroxykaempferol 3-*O*- $\beta$ -D-glucoside (hhw10), 6-hydroxykaempferol 3-*O*- $\beta$ -D-rutinoside (hhw11), kaempferol 3-*O*- $\beta$ -D-sophoroside (hhw12), kaempferol 3-*O*- $\beta$ -D-rutinoside (hhw14), anhydrosafflor yellow B (hhw16), 6-hydroxykaempferol 3,6-di-*O*- $\beta$ -D-glucoside (hhw19), 6-hydroxykaempferol 3-*O*- $\beta$ -rutinoside-6-*O*- $\beta$ -D-glucoside (hhw20), (2*S*)-4',5-dihydroxyl-6,7-di-*O*- $\beta$ -D-glucopyranosyl flavanone (hhw21), quercetin 3-*O*- $\beta$ -D-rutinoside (hhw29), and quercetin. Origins of plants from which compounds were extracted and chemical structures of compounds used in this study are shown in Table 1 and Fig. 1, respectively.

Binding activities of these compounds to the C106 region of DJ-1 were first examined by using a quartz crystal microbalance in which each compound was fixed on a sensor chip, and recombinant wild-type DJ-1, C106S DJ-1, or BSA was applied (Fig. 2). C106S DJ-1 is a substitution mutant in which a cysteine residue at amino acid number 106 was changed to serine, resulting in loss of all DJ-1 activities, including anti-oxidative



**Fig. 1.** Chemical structures of compounds used in this study.



**Fig. 2.** Identification of DJ-1-binding compounds. A: Binding of compounds to wild-type DJ-1, C106S DJ-1, and BSA was examined by using a quartz crystal microbalance as described in Materials and Methods.  $\Delta F$  indicates decreased frequency of a sensor chip. B: Summary of binding activity of compounds to wild-type DJ-1, C106S DJ-1, and BSA.

stress function (11 – 13). The results are summarized in Fig. 2B. Of the 12 compounds tested, 5 compounds, hhw14, hhw16, hhw19, hhw21, and quercetin, bound to wild-type DJ-1 but not to C106S DJ-1. Kaempferol did not bind to DJ-1 (data not shown) and none of the compounds bound to BSA (Fig. 2A).

#### Effects of DJ-1-binding compounds on oxidative stress-induced cell death

Effects of the compounds on oxidative stress-induced cell death were examined. Rat PC12 cells were incubated with 1  $\mu$ M of each compound for 10 min and then treated with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h, and cell viability was measured by an MTT assay (Fig. 3A). Since quercetin has been used at relatively high concentrations for inflammation therapy, 1 and 50  $\mu$ M quercetin were used in this study.

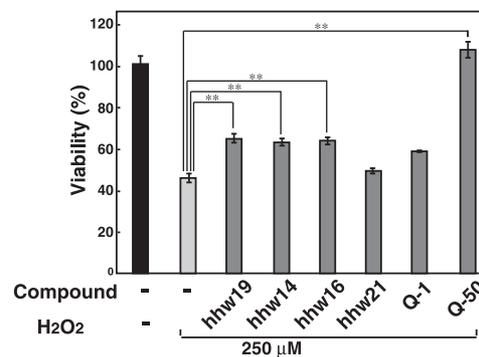
Without compounds, about 58% of the cells died. With 1  $\mu$ M hhw19, hhw14, or hhw16 or 50  $\mu$ M of quercetin, on the other hand, cell death induced by addition of H<sub>2</sub>O<sub>2</sub> was significantly inhibited. Compound hhw21 or 1  $\mu$ M quercetin had no effect (Fig. 3A). It should be noted that all of the compounds at doses used in this study had no toxicity against cultured cells.

Primary neuronal cells of the ventral mesencephalon were prepared from rat embryos on the 17th – 19th days of gestation. Since DJ-1 is expressed both in neurons and glia (22 – 26) and some secreted factors or DJ-1 from astrocytes protected neurons from oxidative stress-induced cell death (25, 26), we used cells cultured for 3 days in which both neurons and glia cells were presented. These cells were pretreated with 1  $\mu$ M of each compound for 10 min and then treated with 150  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h. The results showed that all of the compounds except for hhw21 significantly reduced cell death (Fig. 3B).

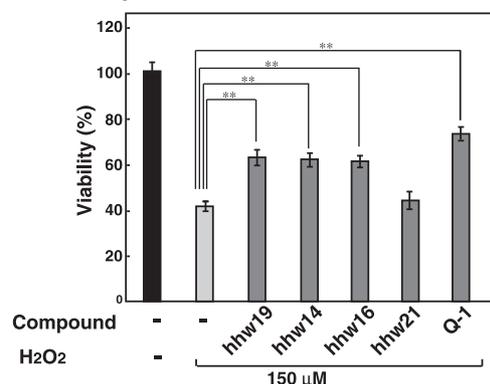
#### DJ-1-specific reaction of DJ-1-binding compounds

To determine the specificity of the DJ-1-binding compounds, their effects on oxidative stress-induced cell death were examined using DJ-1-knockdown cells. First, PC12 cells were transfected with siRNA targeting the rat *DJ-1* (si-DJ-1) or with lipofectamine 2000 alone (vehicle control). At 48 h after transfection, the expression levels of DJ-1 in non-transfected (vehicle control) and si-DJ-1-transfected PC12 cells were examined by Western blotting with an anti-DJ-1 antibody and quantified by normalization of the level of DJ-1 compared to that of actin (Fig. 4A). The results showed that about 40% of DJ-1 expression was knocked down in si-DJ-1-transfected PC12 cells. These cells were treated with 1  $\mu$ M of each compound for 10 min and then with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h, and their viability

#### A. PC12



#### B. Primary neuron

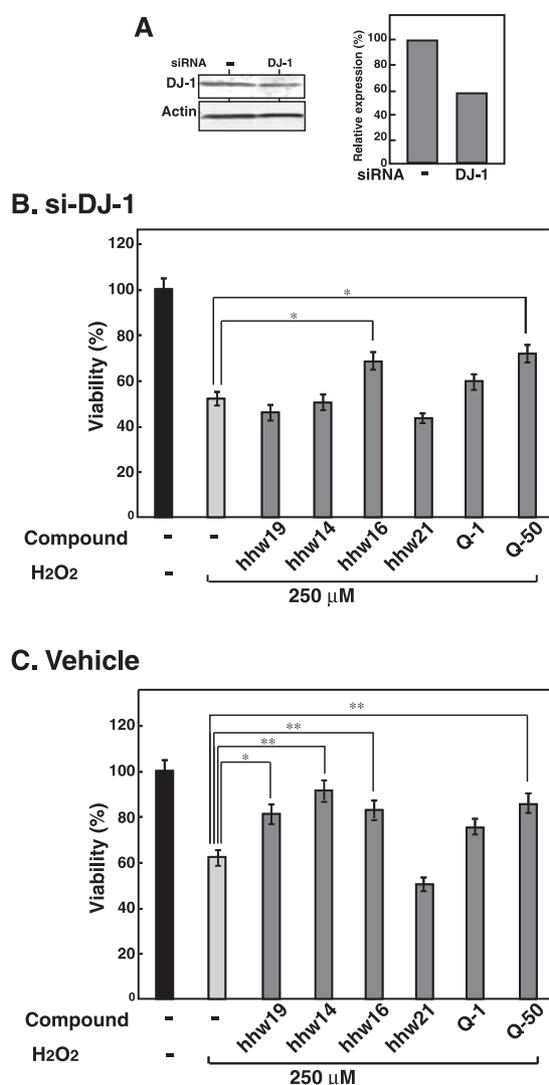


**Fig. 3.** Effects of DJ-1-binding compounds on oxidative stress-induced cell death. A: PC12 cells were pretreated with 1  $\mu$ M of each compound for 10 min and then treated with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h, and cell viability was measured by an MTT assay. Significance: \*\* $P$ <0.01 vs. without compounds. “Q-1” and “Q-50” indicate 1 and 50  $\mu$ M quercetin, respectively. B: Rat mesencephalic cultured neurons were treated with 1  $\mu$ M of each compound for 10 min and then with 150  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h, and cell viability was measured by an MTT assay. Significance: \*\* $P$ <0.01 vs. without compounds.

was examined by an MTT assay (Fig. 4: B and C). Protective activities of all of the compounds except for hhw21 and quercetin against H<sub>2</sub>O<sub>2</sub>-induced cell death were again observed in vehicle control cells (Fig. 4C). In si-DJ-1-transfected cells, on the other hand, compounds hhw19 and hhw14, but not hhw16 and 50  $\mu$ M quercetin, lost their protective activity against H<sub>2</sub>O<sub>2</sub>-induced cell death, indicating that compounds hhw19 and hhw14 function in a DJ-1-dependent manner and that hhw16 and quercetin show their activity in a DJ-1-independent manner. These results also indicate that there is a threshold amount of DJ-1 for DJ-1-binding compounds to function in cells.

#### Effects of DJ-1-binding compounds on production of reactive oxygen species and tyrosine hydroxylase activity

PC12 cells were transfected with siRNA targeting the rat *DJ-1* gene or with lipofectamine 200 alone. At



**Fig. 4.** DJ-1-specific action of DJ-1-binding compounds. A: PC12 cells were transfected with siRNA targeting the rat *DJ-1*. At 48 h after transfection, cell extracts were prepared and expression levels of rat DJ-1 and actin were analyzed by Western blotting with the respective antibodies. After membranes were reacted with the respective secondary antibodies, bands were visualized and their intensities were quantified using an infrared imaging system (Odyssey, LI-COR). Intensity of the band corresponding to DJ-1 in cells without transfection was set to 100. B, C: PC12 cells were transfected with siRNA targeting the rat *DJ-1* (B) and with lipofectamine alone (C). At 48 h after transfection, PC12 cells were treated with 1  $\mu$ M of each compound for 10 min and then with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h, and cell viability was measured by an MTT assay. Significance: \* $P$ <0.05, \*\* $P$ <0.01 vs. without compounds. “Q-1” and “Q-50” indicate 1 and 50  $\mu$ M quercetin, respectively.

48 h after transfection, cells were pretreated with the compounds for 10 min and then with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h, and the levels of ROS in cells were examined after reaction of DCFH by microscopic observation (Fig. 5). Treatment of cells with H<sub>2</sub>O<sub>2</sub> alone resulted in the production of a large amount of ROS both in

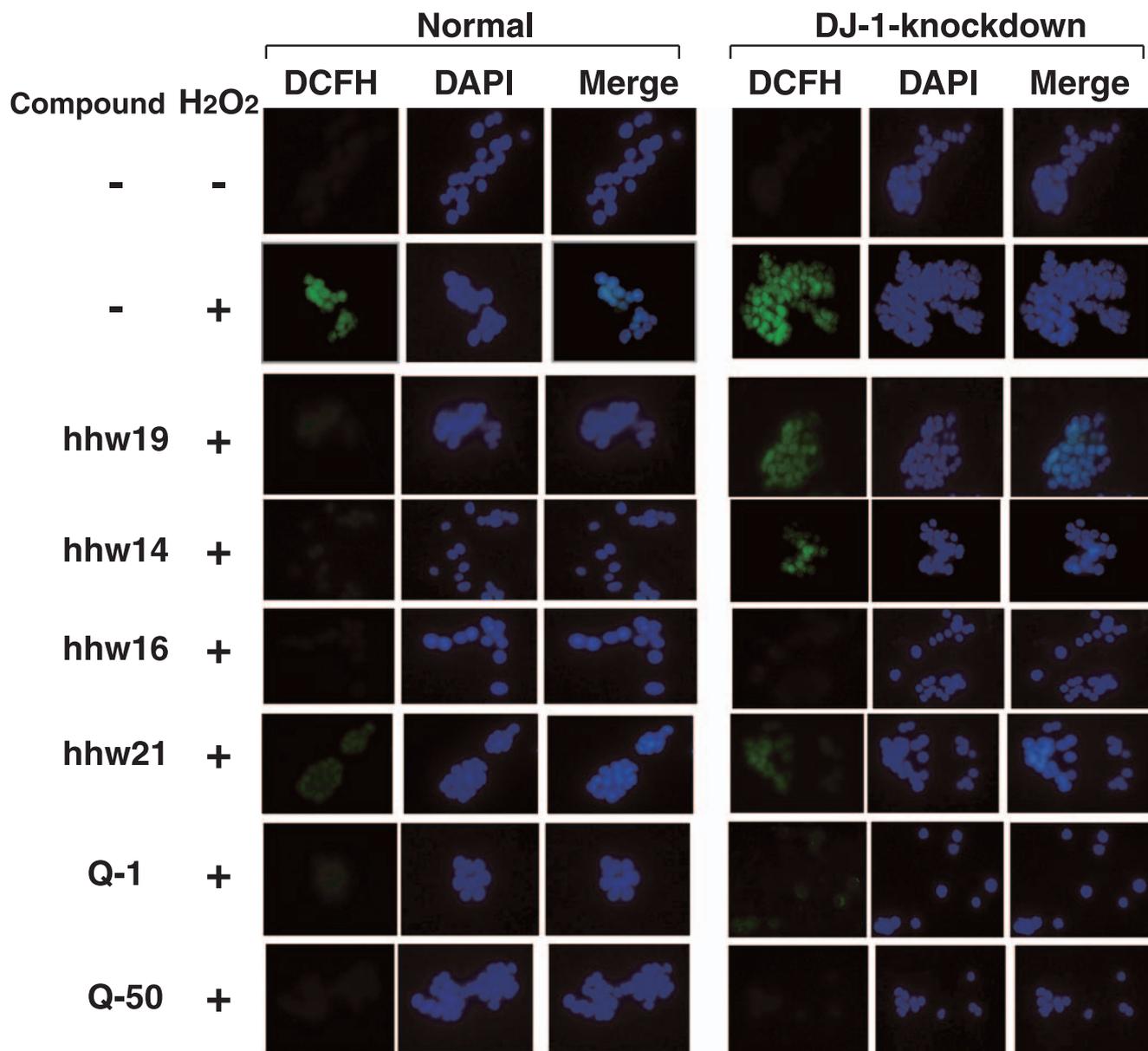
vehicle-transfected (normal) and si-DJ-1-transfected cells (Fig. 5, lanes in “DCFH”). Since all of the compounds are derived from plants that are known to have anti-oxidant activity, pretreatment with all of the compounds, except for hhw21, almost completely abrogated production of ROS in non-transfected cells. In si-DJ-1-transfected cells, on the other hand, compounds hhw19, hhw14, and hhw21 lost their inhibitory activity against production of ROS, and other compounds still abrogated production of ROS. These results indicate that compounds hhw19 and hhw14 exerted their activity in a DJ-1-dependent manner, which is consistent with results for the protective activities against H<sub>2</sub>O<sub>2</sub>-induced cell death as shown in Fig. 4.

Tyrosine hydroxylase (TH) is a key enzyme of dopamine biosynthesis, and TH activity is reduced in patients with PD. When PC12 cells were treated with 150  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h, TH activity was reduced (Fig. 6). Pretreatment of cells with compounds for 10 min restored TH activity (Fig. 6), but the vehicle (DMSO) alone had no effect (data not shown). In this case, all of the compounds stimulated TH activity more than that in cells without treatment of compounds and H<sub>2</sub>O<sub>2</sub>, suggesting that TH activity suppressed by ROS that had been constitutively produced in cells was restored by these compounds.

## Discussion

By using a quartz crystal microbalance (QCM), we identified 5 DJ-1-binding compounds that had been extracted from TCM possessing anti-oxidant activities. Wild-type DJ-1 but not C106S mutant DJ-1 bound to the compounds. Four of the 5 compounds prevented oxidative stress-induced neuronal cell death, reduced the level of ROS, and restored TH activity that had been compromised by oxidative stress. Of those 4 compounds, however, only 2 compounds exerted preventive activity against oxidative stress in a DJ-1-dependent manner and the other two compounds showed activity in a DJ-1-independent manner. Quercetin has, for instance, frequently been used as a supplement for its anti-platelet aggregation, anti-cancer, anti-obesity, and anti-diabetes properties, effects that are based on the anti-oxidant activity of quercetin (27, 28). Although quercetin strongly binds to the C106 region of wild-type DJ-1, it protected cells from H<sub>2</sub>O<sub>2</sub>-induced cell death in a DJ-1-independent manner, suggesting that quercetin targets an unknown molecule(s) that leads to the anti-oxidative stress reaction. These results suggest that although molecular mechanisms of the anti-oxidative stress action of TCM have not been clarified, some TCM target DJ-1.

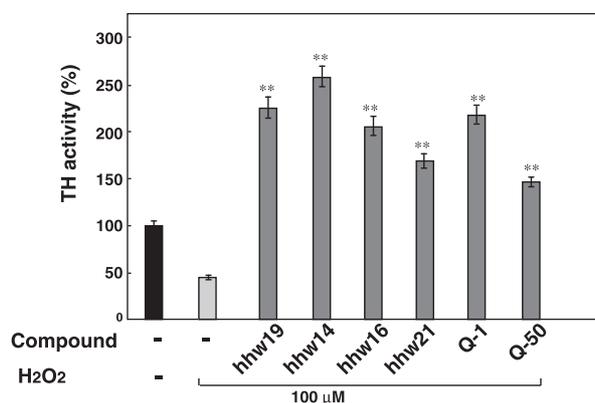
Of the two compounds specific to DJ-1, kaempferol



**Fig. 5.** Effects of DJ-1-binding compounds on production of ROS. PC12 cells were transfected with siRNA targeting the rat *DJ-1* gene (DJ-1-knockdown) or with lipofectamine 2000 alone (Normal). At 48 h after transfection, PC12 cells were treated with 1  $\mu$ M of each compound for 10 min and then with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h. Cells were stained with DAPI and incubated with 5  $\mu$ M DCFH-DA for 10 min at 37°C and visualized under a scanning microscope (Bioevo BZ-9000, Keyence). “Q-1” and “Q-50” indicate 1 and 50  $\mu$ M quercetin, respectively.

3-*O*- $\beta$ -D-rutinoside (hhw14) and 6-hydroxykaempferol 3,6-di-*O*- $\beta$ -D-glucoside (hhw19) are kaempferol derivatives. Kaempferol is quercetin containing an additional OH in one of its benzene rings (Fig. 1). When hhw14 and hhw19 are compared with quercetin and kaempferol derivatives that reacted in a DJ-1-independent manner, bindings of side-chains present in hhw14 and hhw19 to DJ-1 may fill the space that is formed between cysteine 106 and histidine 142, like DJ-1-binding compounds A and B as described previously (18), thereby giving the

specificity of hhw14 and hhw19 to DJ-1. Although hhw21 bound to wt-DJ-1 with the similar potency as hhw14 and hhw19, it lacked anti-oxidative effects. Structural modifications have been shown to affect the biological activities of flavonoids. It has been reported that OH substitutions are important in the anti-oxidant activities of flavonoids (29). Studies of structure and inhibitory activity on farnesyl protein transferase (FPTase) indicate that the number, position, and substitution of OH groups of A and B rings of flavonoids



**Fig. 6.** Effects of DJ-1-binding compounds on TH activity. PC12 cells were treated with 1  $\mu$ M of each compound for 10 min and then with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 3 h. After cell extracts had been prepared, TH activity was measured as described in Materials and Methods. Significance: \*\* $P < 0.01$  vs. without compounds. “Q-1” and “Q-50” indicate 1 and 50  $\mu$ M quercetin, respectively.

and unsaturation of the C2–C3 bond are important factors affecting inhibition of FPTase by flavonoids (30). It has been reported that OH groups, below three or above four, had no effects on the ocular blood flow (31). It appears that three OH groups in the flavonoids are the best to increase the ocular blood flow. Compared to hhw14 and hhw19 that belong to compounds with three OH groups, hhw21 only possesses two OH groups and lacks an OH group at the 7 position and the C2–C3 unsaturation bond, suggesting lack of anti-oxidative effects. A structure similar to that of kaempferol may play an important role in helping these compounds bind to DJ-1. However, the detailed mechanism underlying the different pharmacological effects of DJ-1-binding compounds should be investigated.

In general, natural flavonoids are widely known to possess scavenging properties against oxygen radicals in vivo and in vitro. These compounds themselves would, therefore, have the activity of scavenging ROS. However, due to different substitutions and concentration of flavonoid, not all the flavonoid compounds can show significant scavenging activity of ROS in experiments. Since the compounds used in this study, with the exception of quercetin, are newly found ones, there is yet no information about whether they react with H<sub>2</sub>O<sub>2</sub>. Furthermore, Gao et al. reported that although quercetin and baicalein, two major flavonoids in *Scutellaria baicalensis Georgi*, were oxidized with high concentrations of H<sub>2</sub>O<sub>2</sub> such as 100 mM, they were rather stable and not oxidized by relatively low concentrations of H<sub>2</sub>O<sub>2</sub> such as 10 mM (32). When quercetin and baicalein are treated with 10 mM H<sub>2</sub>O<sub>2</sub>, a sign that they are oxidized is the observed significant decrease in absorbance

at 358 nm and 375 nm, for baicalein and quercetin, respectively. In this study, we used 100–250  $\mu$ M H<sub>2</sub>O<sub>2</sub> and no difference in the absorbance of compounds, including quercetin, was observed in the absence or presence of H<sub>2</sub>O<sub>2</sub> (data not shown), suggesting that the compounds themselves have no scavenging activity of ROS by self-oxidation.

Activity of TH is known to be regulated by several points, including phosphorylation, S-nitrosylation, and/or oxidation of TH. ROS and other signals may affect these modifications of TH. Although hhw21 had weak scavenging activity of ROS, hhw21 significantly stimulated TH activity. While the scavenging activity of ROS of 1  $\mu$ M quercetin was weaker than that of 50  $\mu$ M quercetin, 1  $\mu$ M quercetin stimulated TH activity more strongly than 50  $\mu$ M quercetin. Although the reasons for these differences are not clear at present, effects of these compounds on modifications of TH and changes of signaling pathways in dopamine synthesis should be examined. Since oxidative stress is thought to trigger the onset of various neurodegenerative diseases, these compounds may be useful for treatment of neurodegenerative diseases other than PD.

#### Acknowledgments

We thank Yoko Misawa and Kiyomi Takaya for their technical assistance. This work was supported by grants-in-aid from the Ministry of Education, Science, Culture, and Sports of Japan; the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) in Japan; the National Program for Key Basic Research Projects (No.2004CB518902) of China; National Basic Research Program of China (973 program); and China Scholarship Council (CSC).

#### References

- Li Q, Zhao D, Bezdard E. Traditional Chinese medicine for Parkinson's disease: a review of Chinese literature. *Behav Pharmacol.* 2006;17:403–410.
- Chiu J, Yau T, Epstein RJ. Complications of traditional Chinese /herbal medicines (TCM)-a guide for perplexed oncologists and other cancer caregivers. *Support Care Cancer.* 2009;17:231–240.
- Zhuo Q, Yang X, Wu T, Liu G, Zhou L. Tongxinluo capsule for acute stroke. *Cochrane Database Syst Rev.* 2008;CD004584.
- Nagakubo D, Taira T, Kitaura H, Ikeda M, Tamai K, Iguchi-Aruga SM, et al. DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. *Biochem Biophys Res Commun.* 1997;231:509–513.
- Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science.* 2003; 299:256–259.
- Takahashi K, Taira T, Niki T, Seino C, Iguchi-Aruga SM, Ariga H. DJ-1 positively regulates the androgen receptor by impairing the binding of PIAS $\alpha$  to the receptor. *J Biol Chem.* 2001;276:37556–37563.

- 7 Niki T, Takahashi-Niki K, Taira T, Iguchi-Arigo SM, Ariga H. DJ-1-binding protein, negatively regulates the androgen receptor by recruiting histone deacetylase complex, and DJ-1 antagonizes this inhibition by abrogation of this complex. *Mol Cancer Res.* 2003;1:247–261.
- 8 Zhong N, Kim CY, Rizzu P, Geula C, Porter DR, Pothos EN, et al. DJ-1 transcriptionally up-regulates the human tyrosine hydroxylase by inhibiting the sumoylation of pyrimidine tract-binding protein-associated splicing factor. *J Biol Chem.* 2006; 281:20940–20948.
- 9 Taira T, Saito Y, Niki T, Iguchi-Arigo SM, Takahashi K, Ariga H. DJ-1 has a role in anti-oxidative stress to prevent cell death. *EMBO Rep.* 2004;5:213–218.
- 10 Yokota T, Sugawara K, Ito K, Takahashi R, Ariga H, Mizusawa H. Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition. *Biochem Biophys Res Commun.* 2003;312:1342–1348.
- 11 Canet-Aviles RM, Wilson MA, Miller DW, Ahmad R, McLendon C, Bandyopadhyay S, et al. The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfenic acid-driven mitochondrial localization. *Proc Natl Acad Sci U S A.* 2004;101:9103–9108.
- 12 Martinat C, Shendelman S, Jonason A, Leete T, Beal MF, Yang L, et al. Sensitivity to oxidative stress in DJ-1-deficient dopamine neurons: an ES- derived cell model of primary parkinsonism. *PLoS Biol.* 2004;2:e327.
- 13 Takahashi-Niki K, Niki T, Taira T, Iguchi-Arigo SM, Ariga H. Reduced anti-oxidative stress activities of DJ-1 mutants found in Parkinson's disease patients. *Biochem Biophys Res Commun.* 2004;320:389–397.
- 14 Zhou W, Zhu M, Wilson MA, Petsko GA, Fink AL. The oxidation state of DJ-1 regulates its chaperone activity toward alpha-synuclein. *J Mol Biol.* 2006;356:1036–1048.
- 15 Bandopadhyay R, Kingsbury AE, Cookson MR, Reid AR, Evans IM, Hope AD, et al. The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. *Brain.* 2004; 127:420–430.
- 16 Choi J, Sullards MC, Olzmann JA, Rees HD, Weintraub ST, Bostwick DE, et al. Oxidative damage of DJ-1 is linked to sporadic Parkinson and Alzheimer diseases. *J Biol Chem.* 2006;281:10816–10824.
- 17 Inden M, Taira T, Kitamura Y, Yanagida T, Tsuchiya D, Takata K, et al. PARK7 DJ-1 protects against degeneration of nigral dopaminergic neurons in Parkinson's disease rat model. *Neurobiol Dis.* 2006;24:144–158.
- 18 Yanagisawa D, Kitamura Y, Inden M, Takata K, Taniguchi T, Morikawa S, et al. DJ-1 protects against neurodegeneration caused by focal cerebral ischemia and reperfusion in rats. *J Cereb Blood Flow Metab.* 2008;28:563–578.
- 19 Miyazaki S, Yanagida T, Nunome K, Ishikawa S, Inden M, Kitamura K, et al. DJ-1-binding compounds prevent oxidative stress-induced cell death and movement defect in Parkinson's disease model rats. *J Neurochem.* 2008;105:2418–2434.
- 20 Yanagida T, Kitamura Y, Yamane K, Takahashi K, Takata K, Yanagisawa D, et al. Protection against oxidative stress-induced neurodegeneration by a modulator for DJ-1, a wild-type of familial Parkinson's disease-linked PARK7. *J Pharmacol Sci.* 2009;109:463–468.
- 21 Sawada H, Kohno R, Kihara T, Izumi Y, Sakka N, Ibi M, et al. Proteasome mediates dopaminergic neuronal degeneration, and its inhibition causes  $\alpha$ -Synuclein inclusions. *J Biol Chem.* 2004;279:10710–10719.
- 22 Bandopadhyay R, Kingsbury AE, Cookson MR, Reid AR, Evans IM, Hope AD, et al. The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. *Brain.* 2004; 127:420–430.
- 23 Bader V, Ran Zhu X, Lübbert H, Stichel CC. Expression of DJ-1 in the adult mouse CNS. *Brain Res.* 2005;1041:102–111.
- 24 Yanagida T, Takada K, Inden M, Kitamura Y, Taniguchi T, Yoshimoto K, et al. Distribution of DJ-1, Parkinson's disease-related protein PARK7, and its alteration in 6-hydroxy-dopamine-treated hemiparkinsonian rat brain. *J Pharmacol Sci.* 2006;102:243–247.
- 25 Mullett SJ, Hinkle DA. DJ-1 knock-down in astrocytes impairs astrocyte-mediated neuroprotection against rotenone. *Neurobiol Dis.* 2009;33:28–36.
- 26 Yanagida T, Tsushima J, Kitamura Y, Yanagisawa D, Takata K, Shibaie T, et al. Oxidative stress induction of DJ-1 protein in reactive astrocytes scavenges free radicals and reduces cell injury. *Oxid Med Cell Long.* 2009;1:36–42.
- 27 Singh D, Chander V, Chopra K. The effect of quercetin, a bioflavonoid on ischemia/reperfusion induced renal injury in rats. *Arch Med Res.* 2004;35:484–494.
- 28 Choi EJ, Bae SM, Ahn WS. Antiproliferative effects of quercetin through cell cycle arrest and apoptosis in human breast cancer MDA-MB-453 cells. *Arch Pharm Res.* 2008;31:1281–1285.
- 29 Gille JJ, Joenje H. Cell culture models for oxidative stress: Superoxide and hydrogen peroxide versus normobaric hyperoxia. *Mutat Res.* 1992;275:405–414.
- 30 Cantoni O, Cattabeni F, Stocchi V, Meyn RE, Cerutti P, Murray D. Hydrogen peroxide insult in cultured mammalian cells: Relationships between DNA single-strand breakage, poly(ADP-ribose)metabolism and cell killing. *Biochim Biophys Acta.* 1989;1014:1–7.
- 31 Wink DA, Cook JA, Pacelli R, DeGraff W, Gamson J, Liebmann J, et al. The effect of various nitric oxidizedonor agents on hydrogen peroxide-mediated toxicity: A direct correlation between nitric oxide formation and protection. *Arch Biochem Biophys.* 1996;331:241–248.
- 32 Gao Z, Huang K, Xu H. Protective effects of flavonoids in the roots of *Scutellaria baicalensis* Georgi against hydrogen peroxide-induced oxidative stress in HS-SY5Y cells. *Pharmacol Res.* 2001;43:173–178.
- 33 Wei X, Liu H, Sun X, Fu F, Zhang X, Wang J, et al. Hydroxysafflor yellow A protects rat brains against ischemia-reperfusion injury by antioxidant action. *Neurosci Lett.* 2005;386:58–62.
- 34 Zhu H, Wang Z, Ma C, Tian J, Fu F, Li C, et al. Neuroprotective effects of hydroxysafflor yellow A: in vivo and in vitro studies. *Planta Med.* 2003;69:429–433.
- 35 Ye SY, Gao WY. Hydroxysafflor yellow A protects neuron against hypoxia injury and suppresses inflammatory responses following focal ischemia reperfusion in rats. *Arch Pharm Res.* 2008;31:1010–1015.
- 36 Wang C, Zhang D, Li G, Liu J, Tian J, Fu F, et al. Neuroprotective effects of safflor yellow B on brain ischemic injury. *Exp Brain Res.* 2007;177:533–539.
- 37 Rigano D, Formisano C, Basile A, Lavitola A, Senatore F, Rosselli S, et al. Antibacterial activity of flavonoids and phenylpropanoids from *Marrubium globosum* ssp. *Libanoticum*. *Phytother Res.* 2007;21:395–397.