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Brain Research 965 (2003) 130–136
BRAIN RESEARCH
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Research report

Neuroprotective effects of antioxidative flavonoids, quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether, isolated from *Opuntia ficus-indica* var. *saboten*

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Accepted 4 December 2002

Abstract

The flavonoids quercetin, (+)-dihydroquercetin, and quercetin 3-methyl ether were isolated from the ethyl acetate fractions of the fruits and stems of *Opuntia ficus-indica* var. *saboten*. In the present study, we evaluated their protective effects against oxidative neuronal injuries induced in primary cultured rat cortical cells and their antioxidant activities by using three different cell-free bioassays. Quercetin was found to inhibit H_2O_2- or xanthine (X)/xanthine oxidase (XO)-induced oxidative neuronal cell injury, with an estimated IC_{50} of 4–5 μg/ml. However, it was no more protective at concentrations of 30 μg/ml and above. (+)-Dihydroquercetin concentration-dependently inhibited oxidative neuronal injuries, but it was less potent than quercetin. On the other hand, quercetin 3-methyl ether potently and dramatically inhibited H_2O_2- and X/XO-induced neuronal injuries, with IC_{50} values of 0.6 and 0.7 μg/ml, respectively. All three principles markedly inhibited lipid peroxidation and scavenged 1,1-diphenyl-2-picrylhydrazyl free radicals. In addition, quercetin and quercetin 3-methyl ether were shown to inhibit XO activity in vitro, with respective IC_{50} values of 10.67 and 42.01 μg/ml. These results indicate that quercetin, (+)-dihydroquercetin, and quercetin 3-methyl ether are the active antioxidant principles in the fruits and stems of *Opuntia ficus-indica* var. *saboten* exhibiting neuroprotective actions against the oxidative injuries induced in cortical cell cultures. Furthermore, quercetin 3-methyl ether appears to be the most potent neuroprotectant of the three flavonoids isolated from this plant.

Theme: Development and regeneration

Topic: Neuronal death

Keywords: *Opuntia ficus-indica* var. *saboten*; Neuroprotection; Oxidative injury; Cortical neuron; Antioxidant; Quercetin; Quercetin 3-methyl ether; (+)-Dihydroquercetin

1. Introduction

Opuntia ficus-indica var. *saboten* (Cactaceae), a tropical or subtropical plant originally grown in South America, is widely cultivated in Cheju Island in Korea. Its fruits and stems have been traditionally used as oriental folk medicine for burns, wounds, edema, bronchial asthma, diabetes, and indigestion [1]. It has been reported that the extracts of fruits and stems exhibit hypoglycemic [13,28], anti-ulcer [10,18], and anti-allergic actions [20]. In addition, Park et al. reported analgesic and anti-inflammatory actions of the fruit and stem extracts [25], and later identified β-sitosterol as the active anti-inflammatory principle from the stem extract [26].

Recently, the methanol extract of the fruits of *Opuntia ficus-indica* var. *saboten* was shown to inhibit free radical-induced neuronal injury in mouse cortical cultures [29]. Given the suggested roles of free radicals in neuronal death after ischemia or trauma and in neurodegenerative disorders [11], we attempted to isolate and identify the active

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0006-8993/02/$ – see front matter
doi:10.1016/S0006-8993(02)04150-1

principles in this plant and to characterize their neuroprotective and antioxidant actions using primary cultured rat cortical cells and three different cell-free assay systems.

In our preliminary study, we observed that, in addition to the fruit extract, the methanol extract of the stems also inhibited the oxidative injury induced by H_2O_2 or xanthine (X)/xanthine oxidase (XO) in cortical cell cultures. Thus, both the methanol extracts of the fruits and stems were further processed to isolate the active principles. The methanol extracts were separately suspended in water and then partitioned consecutively in dichloromethane, ethyl acetate, and butanol. The ethyl acetate fractions were found to inhibit oxidative neuronal injury, whereas the other fractions were inactive. In addition, the inhibition by the ethyl acetate fractions was more potent than that by the respective methanol extract. Ten constituents were isolated from the ethyl acetate fractions and identified by chemical and spectroscopic analyses [19]. These include principles identified previously [15] and newly identified principles in this plant [19]. Among these constituents, three flavonoids, quercetin, (+)-dihydroquercetin, and quercetin 3-methyl ether, were found to protect primary cultured rat cortical cells from H_2O_2- or X/XO-induced oxidative injury. The present study describes and compares the neuroprotective and antioxidant actions of these three flavonoids isolated from the fruits and stems of *Opuntia ficus-indica* var. *saboten*.

2. Materials and methods

2.1. Chemicals and animals

Materials used for cell cultures including minimum essential medium (MEM) were obtained from Gibco BRL. Glucose, Trizma base, X, XO, HEPES, H_2O_2, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 1-methyl-2-phenylindole, and lactate dehydrogenase (LDH) diagnostic kit (Sigma 500) were purchased from Sigma. LPO-586™, used for the lipid peroxidation (LPO) assay, was obtained from Bioxytech (Gagny, France). The fruits and stems of *Opuntia ficus-indica* var. *saboten* were purchased from the Kyongdong herbal drug market (Seoul, Korea). Voucher specimens (901-15) have been deposited at the herbarium in the Korea Institute of Science & Technology. Quercetin (FAB-MS m/z 303 $[M+H]^+$ (calculated for $C_{15}H_{11}O_7$, 303)), (+)-dihydroquercetin ($[\alpha]_D^{23}$ +22.0° (*c* 1.68, MeOH); FAB-MS m/z 305 $[M+H]^+$ (calculated for $C_{15}H_{13}O_7$, 305)), and quercetin 3-methyl ether (FAB-MS m/z 317 $[M+H]^+$ (calculated for $C_{16}H_{13}O_7$, 317)) were isolated as described [19] from the ethyl acetate fractions of the fruits and stems of *Opuntia ficus-indica* var. *saboten*, and identified by using chemical and spectroscopic methods (^1H–^1H COSY, DEPT, HMQC and HMBC). The purities of the isolated compounds were >97% by HPLC analysis. All other chemicals were reagent grade or better.

Timed-pregnant Sprague–Dawley (SD) rats for primary cortical cell cultures and male SD rats (260–280 g) for preparations of brain homogenates were obtained from Daehan Biolink (Chungbuk, Korea), and maintained in a room with a 12 h light cycle at a controlled temperature (22±2 °C) until used. All animal experiments were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Primary cultures of rat cortical cells, experimental treatments, and the assessment of neuronal injuries

Cortical cell cultures containing neuronal and non-neuronal cells were prepared from the cerebral cortices of SD rat embryos at 16–18 days of gestation and maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2, as previously described [5–7]. Cultures were used for experiments 12–14 days after plating.

Oxidative neuronal injuries were induced by the exposures of the cultures to H_2O_2 (100 μM) for 5 min or X (0.5 mM)/XO (10 mU/ml) for 10 min in HEPES-buffered salt solution (HBSS) [17]. After exposure, the cultures were washed and maintained in MEM supplemented with 21 mM glucose for 20–24 h, and neuronal damage was quantified by measuring the activity of LDH released into the culture media. To evaluate the effects of the isolated flavonoids on the oxidative neuronal injuries, the cultures were simultaneously exposed during the oxidative insults to various concentrations of the test principles. Stock solutions of the isolated principles were prepared in 100% DMSO at 200× the highest concentration tested and then serially diluted in HBSS to the desired concentrations. For sham treatment, sister cultures were exposed to 0.5% DMSO, which showed no effect on cell viability [5–7]. The qualitative assessment of neuronal injury was examined by phase-contrast microscopy.

Data were calculated as percentages of the control LDH activity in the culture medium exposed to the respective oxidative insult in the absence of test principles. The mean values from three separate experiments performed in duplicate were analyzed by non-linear regression using Prism (GraphPad Software, USA) to determine the concentrations inhibiting neuronal injuries by 50% (IC_{50} values).

2.3. LPO inhibition assay

SD rat brains were homogenized in 20 mM Tris buffer (pH 7.4) and centrifuged at 3000×*g* for 10 min at 4 °C. The supernatant was then removed and used for the LPO assay. The incubation mixture (final volume 200 μl) contained brain homogenate, 100 μM Fe^{2+}, 400 μM ascorbic acid, and various concentrations of the test principles dissolved in DMSO. LPO was evaluated by

determining the formation of malonaldehyde, the main decomposition product of peroxides derived from polyunsaturated fatty acids [24]. Malonaldehyde reacts at 45 °C during the incubation period of 60 min with the chromogenic reagent *N*-methyl-2-phenylindole to yield a stable chromophore [4], which can be measured at 586 nm in the clear supernatant of the reaction mixture.

2.4. Assay for DPPH radical scavenging activity

The DPPH radical scavenging activities of the isolated flavonoids were tested according to the method previously described [2]. In brief, reaction mixtures containing various concentrations of the test principles dissolved in DMSO and 300 μM DPPH ethanolic solution in a 96-well microtiter plate were incubated at 37 °C for 30 min and absorbance was measured at 515 nm. Percent scavenging activity was determined by comparison with the vehicle-treated control group and calculated according to the equation below [22]. IC_{50} values denote the concentrations required to scavenge 50% of the free radicals generated by DPPH

$$\text{inhibition (\%)} = [(\text{abs}_{\text{control}} - \text{abs}_{\text{sample}})/\text{abs}_{\text{control}}] \times 100.$$

2.5. Assay for inhibition of XO activity

The activity of XO was determined by the method previously described [23]. In brief, 100 μM X solution (980 μl) in sodium phosphate buffer (pH 7.8) and 10 μl of XO solution (0.04 U) were incubated for 5 min at room temperature in the presence of either DMSO or various concentrations of test principles dissolved in DMSO (10 μl). The formation of uric acid from X by XO was measured by recording the optical density at 295 nm for 5 min against a blank containing 10 μl of 100 μM phosphate buffer instead of the enzyme. The IC_{50} values were calculated from the percent inhibition of XO activity, assessed by decreased uric acid formation [21].

3. Results

3.1. Inhibition of oxidative neuronal injuries by quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether

To evaluate the protective effects of the three flavonoids, quercetin, (+)-dihydroquercetin, and quercetin 3-methyl ether (Fig. 1), on oxidative neuronal injury, we employed primary cultured rat cortical cells maintained for 12–14 days in vitro. When the cultures were exposed to 100 μM H_2O_2 for 5 min or X (0.5 mM)/XO (10 mU/ml) for 10 min, prominent neuronal cell death was observed over 20–24 h after the exposure (Fig. 2C and D) compared to the respective sham-treated control cultures (Fig. 2A and B). In agreement with previous reports [8,17,29], ~80–90% cells were found to be damaged based on LDH activity measurements in the culture media. Simultaneous treatments of the cultures during the respective oxidative insults with the isolates at a concentration of 10 μg/ml dramatically inhibited the H_2O_2- or X/XO-induced oxidative injuries (Fig. 2E–J).

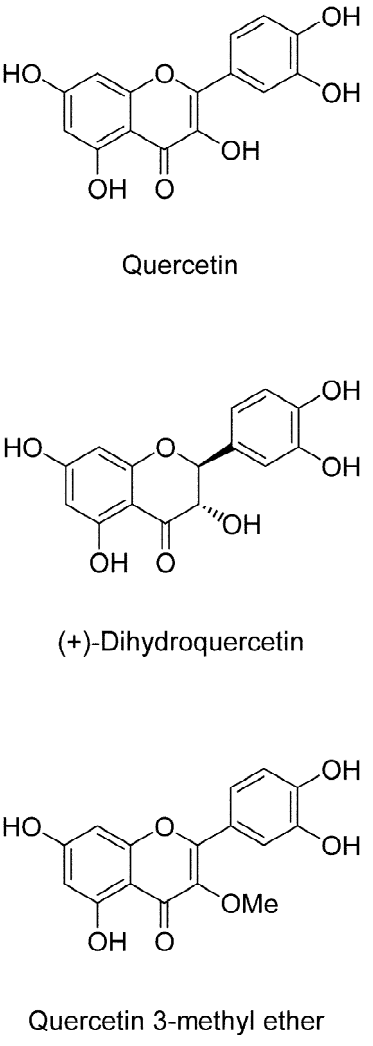

Fig. 1. The structures of quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether.

To assess the neuroprotective potency of the isolates, the cultures were treated with various concentrations of the isolated principles. As illustrated in Fig. 3A, quercetin (3,3′,4′,5,7-pentahydroxyflavone, Fig. 1) exhibited marked inhibition of H_2O_2-induced injury at concentrations of 3 and 10 μg/ml. Similarly, the oxidative injury induced by X/XO was strongly inhibited by quercetin at a concentration of 10 μg/ml (Fig. 3B). The IC_{50} values estimated from these data were 4.1 and 5.5 μg/ml, respectively (Table 1). However, quercetin dramatically increased LDH releases at 30 and 100 μg/ml (Fig. 3A and B), indicating that it is no longer neuroprotective at these concentrations.

The H_2O_2- and X/XO-induced oxidative injuries were

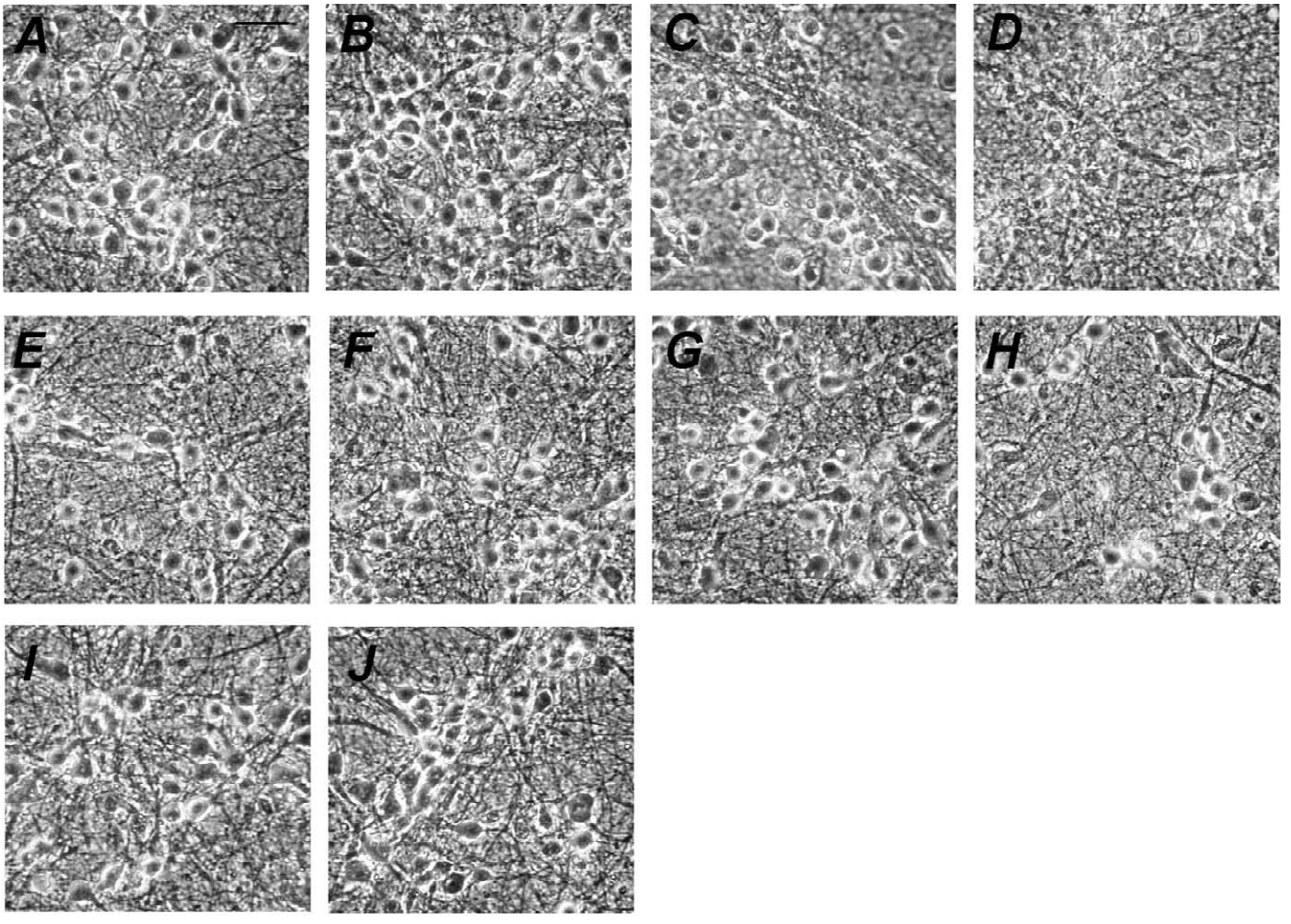

Fig. 2. Protection of rat cortical cells by quercetin, (+)-dihydroquercetin or quercetin 3-methyl ether against oxidative injury. Phase-contrast photomicrographs of primary cultured rat cortical cells (12–14 days after plating) are shown after exposure to either H_2O_2 (100 μM) for 5 min (C, E, G, I) or xanthine (X)/xanthine oxidase (XO) (0.5 mM/10 mU/ml) for 10 min (D, F, H, J) in the absence (C, D) or presence of quercetin (E, F), (+)-dihydroquercetin (G, H) or quercetin 3-methyl ether (I, J) at 10 μg/ml, respectively, followed by incubation for 20–24 h at 37 °C, as described in the Materials and methods. A and B represent respective sham-treated cultures exposed to 0.5% vehicle (DMSO). Scale bar = 50 μm.

also inhibited by (+)-dihydroquercetin (3,3′,4′,5,7-pentahydroxyflavanone, Fig. 1), but it was less potent than quercetin (Fig. 3 and Table 1). On the other hand, quercetin 3-methyl ether (Fig. 1) exhibited the most potent and dramatic inhibitions of H_2O_2- and X/XO-induced oxidative injuries (Fig. 3), with respective IC_{50} values of 0.6 and 0.7 μg/ml (Table 1). In contrast to quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether exhibited concentration-dependent protective effects at the concentrations indicated in Fig. 3. Furthermore, in additional experiments, we found that the protective effects of (+)-dihydroquercetin and quercetin 3-methyl ether were maintained at 300 and 30 μg/ml, respectively (data not shown).

3.2. Inhibition of LPO by quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether

Quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether potently inhibited LPO initiated by $FeCl_2$ in rat brain homogenates. Their respective IC_{50} values were 0.23, 1.02, and 0.74 μg/ml (Table 2).

3.3. DPPH radical scavenging activities of quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether

Quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether were found to effectively scavenge free radicals generated by DPPH. The concentrations exhibiting 50% scavenging effects were 10.37, 14.34, and 14.62 μg/ml, respectively (Table 2).

3.4. Effects of quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether on XO activity

Quercetin and quercetin 3-methyl ether were found to inhibit XO activity, measured in vitro by the formation of uric acid from X. Their respective IC_{50} values were 10.67

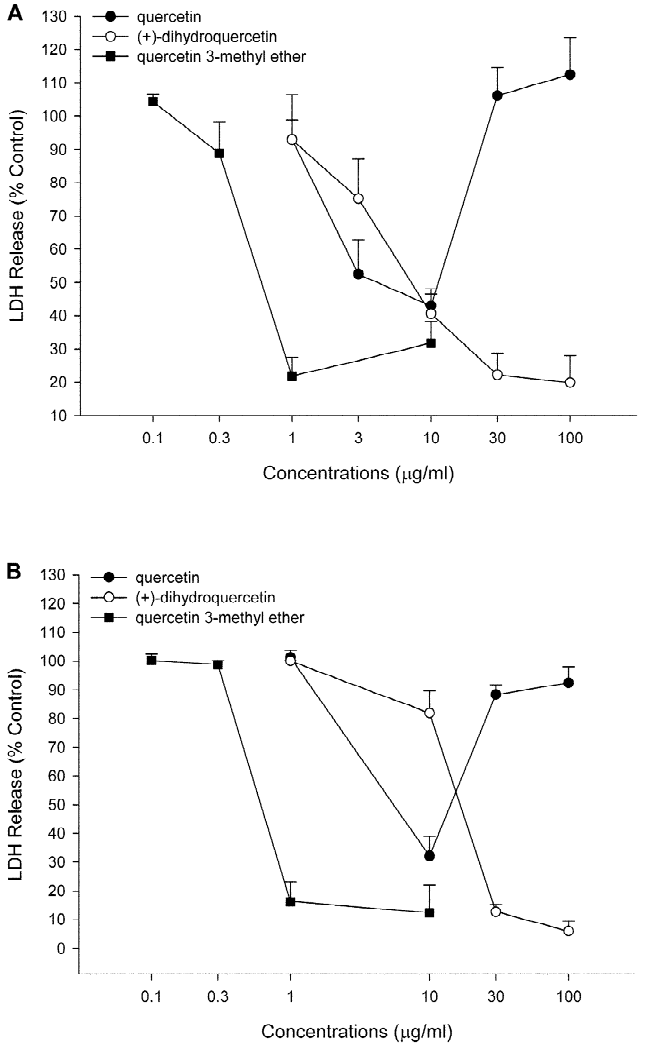

Fig. 3. Inhibition of oxidative neuronal injury by quercetin, (+)-dihydroquercetin or quercetin 3-methyl ether. Primary cultured rat cortical cells (12–14 days after plating) were exposed to H_2O_2 for 5 min (A) or xanthine (X)/xanthine oxidase (XO) for 10 min (B) in the presence of various concentrations of quercetin, (+)-dihydroquercetin or quercetin 3-methyl ether. LDH activities in the culture media were measured after 20–24 h of exposure, as described in the Materials and methods. Data were calculated as percentages of control LDH activity measured in the absence of the test principles. Each point represents the mean±S.D. of three separate experiments performed in duplicate.

Table 1
Neuroprotective effects of quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether on the oxidative injuries induced in primary cultured rat cortical cells

Flavonoids isolated from *Opuntia ficus-indica*	Experimental conditions	
	H_2O_2-induced injury (IC_{50}, μg/ml)[a]	X/XO-induced injury (IC_{50}, μg/ml)[a]
Quercetin	4.1	5.5
(+)-Dihydroquercetin	7.8	16.6
Quercetin 3-methyl ether	0.6	0.7

[a] IC_{50} values, concentrations inhibiting 50% of neuronal injuries, were calculated by nonlinear regression of the data shown in Fig. 3 using GraphPad Prism.

and 42.01 μg/ml (Table 2). In contrast, (+)-dihydroquercetin showed no effect on this enzyme at the concentrations tested in this study.

4. Discussion

Brain is known to be particularly susceptible to oxidative damage because of its high utilization of oxygen, and its high levels of unsaturated lipids and transition metals such as iron, and because of its relatively deficient antioxidative defense mechanisms [27]. Moreover, reactive oxygen radicals and LPO have been implicated in the pathogenesis of a number of neurological insults, including brain trauma, ischemia, and neurodegenerative disorders [11]. Therefore, pharmacological agents capable of scavenging free radicals and/or inhibiting LPO, and thereby, protecting neurons from oxidative injuries may provide useful therapeutic potentials for the prevention or treatment of the neurodegenerative disorders caused by oxidative stress.

During our search for naturally occurring neuroprotective agents, the methanol extract prepared from the fruits of *Opuntia ficus-indica* var. *saboten* was reported to attenuate oxidative radical-induced neuronal injury [29]. The present study demonstrates that the three flavonoids, quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether (Fig. 1), isolated from the fruits and stems of this plant are the active antioxidant principles, which markedly inhibit the neuronal injuries induced by H_2O_2 or X/XO in primary cultured rat cortical cells. The exposure of cultured cortical cells to H_2O_2 or X/XO generates free radicals, such as superoxide and hydroxyl radicals. These radicals are believed to actively participate in the initiation of LPO and eventually cause cell death (Fig. 2C and D) by damaging all types of biomolecules, including proteins, lipids and DNA [11].

Quercetin is one of the most frequently found and studied flavonoids and has many known biological activities, including antioxidant action [9]. Quercetin has been shown in many cell-free experimental systems to scavenge reactive oxygen radicals and to reduce oxidative DNA damage and LPO [3,12]. The present study confirmed the antioxidant effects of quercetin in three different cell-free assay systems and compared to the effects of (+)-dihydroquercetin and quercetin 3-methyl ether. Quercetin was found to more potently inhibit LPO and XO and to have greater radical scavenging activity than the other two flavonoids (Table 2).

Recently, Ishige et al. [14] found in the mouse hippocampal cell line HT-22 that many flavonoids including quercetin protect neuronal cells from oxidative stress induced by glutamate. In HT-22 cells lacking ionotropic glutamate receptors, glutamate inhibits cystine uptake and depletes intracellular glutathione, which leads to the accumulation of reactive oxygen species and ultimately

Table 2
Inhibition of lipid peroxidation (LPO) or xanthine oxidase (XO) by quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether, and their DPPH radical scavenging activities

Flavonoids isolated from *Opuntia ficus-indica*	Experimental conditions		
	LPO (IC_{50}, μg/ml)	XO activity (IC_{50}, μg/ml)	DPPH radicals (IC_{50}, μg/ml)
Quercetin	0.23±0.05	10.67±2.75	10.37±1.53
(+)-Dihydroquercetin	1.02±0.25	ND	14.34±0.36
Quercetin 3-methyl ether	0.74±0.08	42.01±3.12	14.62±1.09

Inhibition of LPO or XO and DPPH radical scavenging activities were determined in the presence of various concentrations of quercetin, (+)-dihydroquercetin or quercetin 3-methyl ether, as described in the Materials and methods. IC_{50} values, concentrations inhibiting 50% of LPO or XO, or scavenging 50% DPPH radicals, were calculated by nonlinear regression using GraphPad Prism. Each point represents the mean±S.D. from three measurements performed in duplicates. ND, not detected.

causes neuronal death [14]. In agreement with this report, we also found in the present study that quercetin protects cultured rat cortical cells from the oxidative injuries induced by free radicals generated by H_2O_2 or X/XO.

However, as we increased the concentrations of quercetin to 30 or 100 μg/ml, it proved to be no longer protective (Fig. 3), implying its intrinsic cytotoxicity or prooxidant activity at these concentrations in cultured cortical cells. Similar findings have been previously reported in Jurkat T-lymphocytes [16], showing that low concentrations such as 10 μM (i.e. 3 μg/ml) of quercetin scavenged free radicals and thereby inhibited oxidative DNA damage, whereas higher concentrations such as 100 μM (i.e. 30 μg/ml) were shown to induce cellular DNA damage. The prooxidant effects of high concentrations of quercetin may be due to the presence of metal ions and the generation of reactive oxygen species [16] or may depend on the redox state of its biological environment [9]. In contrast to quercetin, (+)-dihydroquercetin is shown to be neuroprotective at 100 μg/ml, which is about three times the most effective concentration (Fig. 3). Moreover, the protective effects of (+)-dihydroquercetin were maintained at 300 μg/ml (data not shown). Similarly, quercetin 3-methyl ether retained the protective effects at 30 μg/ml, 30 times the most effective concentration. Based on these findings, it is unlikely that (+)-dihydroquercetin and quercetin 3-methyl ether are cytotoxic or prooxidant at these concentrations in cultured rat cortical cells.

Despite the fact that quercetin exhibited the most potent antioxidant activity in our cell-free assays (Table 2), quercetin 3-methyl ether most potently protected neuronal cells against oxidative injuries in cortical cultures (Table 1, Fig. 3). In fact, quercetin 3-methyl ether was 6–9 times more potent than quercetin, and 13–25 times more potent than (+)-dihydroquercetin in terms of the inhibition of H_2O_2- or X/XO-induced oxidative injury (Table 1). The hydrophobic nature of quercetin 3-methyl ether is thought to be responsible for its potent neuroprotective action in cultured cells. Although the radical scavenging action of quercetin 3-methyl ether and its inhibitory action on XO may contribute, to some extent, to its neuroprotective effects in cortical cultures, its potent inhibition of LPO is thought to better reflect its neuroprotective mechanism, since the IC_{50} value of LPO is within the same concentration range as the IC_{50} values of H_2O_2- or X/XO-induced oxidative injury (Tables 1 and 2). XO catalyzes oxidation of hypoxanthine and xanthine to uric acid whilst reducing O_2 to both superoxide anion and H_2O_2 [23]. As shown in Table 2, relatively high concentrations of quercetin 3-methyl ether are required for the inhibition of XO (IC_{50}=42.01 μg/ml), and thus, it appears to inhibit the neuronal injury induced by radicals, such as the superoxide anion generated by X/XO, rather than to inhibit the generation of radicals during the oxidation of X by XO.

Taken together, these results indicate that the flavonoids quercetin, (+)-dihydroquercetin, and quercetin 3-methyl ether are the active antioxidant principles in the fruits and stems of *Opuntia ficus-indica* var. *saboten*, exhibiting neuroprotective effects against the oxidative injury induced by H_2O_2 or X/XO in primary cultured rat cortical cells. Furthermore, quercetin 3-methyl ether appears to be the most potent and promising neuroprotectant among the three flavonoids isolated from this plant. It is evident that the neuroprotective actions of *Opuntia ficus-indica* var. *saboten* or of its active principles may be beneficial for the prevention and treatment of oxidative stress-induced neurological disorders.

Acknowledgements

This research was supported by grants (PF002103-01 and PF002103-04) from the Plant Diversity Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology in Korea.

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