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Neuroprotective and Antioxidant Effects of the Butanol Fraction Prepared from *Opuntia ficus-indica* var. *saboten*

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Abstract – The fruits and stems of *Opuntia ficus-indica* var. *saboten* have been reported to exhibit a variety of pharmacological actions, including antioxidant, analgesic, anti-inflammatory, and anti-ulcer effects. In the present study, we evaluated effects of the butanol fraction (SK OFB901) prepared from the 50% ethanol extract of the stems on various types of neuronal injuries induced by oxidative stress, excitotoxins, and amyloid β (A_β) in primary cultured rat cortical cells. Its antioxidant and radical scavenging activities were also evaluated by cell-free bioassays. We found that SK OFB901 strongly inhibited the oxidative neuronal damage induced by H_2O_2 or xanthine/xanthine oxidase. In addition, it exhibited marked inhibition of the excitotoxic neuronal damage induced by glutamate, N-methyl-D-aspartic acid, or kainate. Furthermore, the $A_{\beta(25-35)}$-induced neurotoxicity was also significantly attenuated by SK OFB901. It was found to inhibit lipid peroxidation initiated by Fe^{2+} and L-ascorbic acid in rat brain homogenates and scavenge 1,1-diphenyl-2-picrylhydrazyl free radicals. These results indicate that the butanol fraction prepared from the stems of *Opuntia ficus-indica* var. *saboten* exerts potent antioxidant and neuroprotective effects through multiple mechanisms, implying its potential applications for the prevention or management of neurodegenerative disorders associated with oxidative stress, excitotoxicity, and A_β.

Key words □ *Opuntia ficus-indica*, Neuroprotection; Antioxidant, Cortical culture, Excitotoxicity, Amyloid β (A_β)

INTRODUCTION

Opuntia ficus-indica var. *saboten* (Cactaceae), a tropical or subtropical plant originally grown in South-central Mexico, is now commercially cultivated in many warm areas of the world including Jeju Island in Korea (Ahn, 1998; Feugang *et al.*, 2006). It has been traditionally used in oriental folk medicine to treat diabetes, bronchial asthma, burns, and indigestion (Ahn, 1998). The fruits and stems of this plant have been reported to exhibit a variety of pharmacological actions, including antioxidant (Lee *et al.*, 2002; Galati *et al.*, 2003; Stintzing *et al.*, 2005), analgesic (Park *et al.*, 1998), anti-inflammatory (Park *et al.*, 1998, 2001), hypoglycemic (Frati *et al.*, 1990; Galati *et al.*, 2003), and anti-ulcer (Galati *et al.*, 2002, 2007; Lee *et al.*, 2002) effects. Based on these previous reports and more recent findings of several active components and their multifunctional properties, the fruits and stems have received much attention and been recognized as valuable sources of health-promoting food and medicine (Stintzing and Reinhold, 2005; Feugang *et al.*, 2006).

Recently, additional pharmacological action of the fruits and stems has been described. The methanol extract of the fruits was found to inhibit neuronal injuries induced in mouse cortical cultures by radicals, N-methyl-D-aspartic acid (NMDA), kainic acid (KA), or oxygen-glucose deprivation (Wie, 2000; Kim *et al.*, 2006). Its neuroprotective action was further confirmed in an animal model of global ischemia, showing moderately reduced neuronal damage in the hippocampal CA1 region of the gerbils pretreated with the fruit methanol extract (Kim *et al.*, 2006). In a parallel study, the methanol extract of the stems was also shown to exhibit mild neuroprotective actions, inhibiting the H_2O_2-induced oxidative damage of the cultured rat cortical cells (Dok-Go *et al.*, 2003). Among the constituents isolated from the subsequent partitioning of the methanol

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extracts of the fruits and stems, the three flavonoids, quercetin, (+)-dihydroquercetin, and quercetin 3-methyl ether, were identified as active principles (Dok-Go *et al.*, 2003). Besides these flavonoids, other constituents such as ascorbic acid, betalain pigments and phenolics may also attribute to the neuroprotective actions (Stintzing *et al.*, 2005).

In an effort to obtain a preparation exhibiting improved pharmacological profiles, the butanol fraction (SK OFB901) was prepared from 50% ethanol extract of the stems. SK OFB901 has been recently described to inhibit NO production in lipopolysaccharide (LPS)-activated microglia and scavenge peroxynitrite (Lee *et al*., 2006). To further characterize pharmacological properties of SK OFB901 and compare with the actions of the methanol extract, we investigated in this study its effects on various types of neuronal injuries induced by oxidative stress, excitotoxins, and amyloid β (A_β) using primary cultured rat cortical cells. Its antioxidant and radical scavenging activities were also evaluated by cell-free bioassay systems.

MATERIALS AND METHODS

Materials

Materials used for cell cultures including minimum essential medium (MEM) and fetal calf serum were obtained from Gibco BRL (Grand Island, NY, USA). NMDA, L-glutamate, xanthine, xanthine oxidase, H_2O_2, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). $A_{\beta(25-35)}$ was obtained from Bachem Ltd. (San Carlos, UK) and KA from Tocris Bioscience (Bristol, UK). Culture plates were from Falcon (Franklin Lakes, NJ, USA), and all other chemicals were of reagent grade or higher.

Animals

Timed-pregnant Sprague-Dawley (SD) rats for primary cultures and male SD rats for preparations of brain homogenates were obtained from Daehan Biolink (Chungbuk, Korea). Animals were maintained with Purina laboratory chow and water *ad libitum* in our animal facility with a 12 h light cycle at a controlled temperature (22±2ºC) until used. All animal experiments were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Preparation of SK OFB901

The stems of *Opuntia ficus-indica* var. *saboten* cultivated in Jeju Island, Korea, were collected in March 2004 and authenticated by Professor K. S. Yang, Division of Pharmacy, Sookmyung Women's University. A voucher specimen (901-15-2004) has been deposited in the herbarium of the Korea Institute of Science and Technology. SK OFB901 was prepared as previously described (Lee *et al.*, 2006). In brief, the dried stems (1 kg) were extracted with 50% (v/v) ethanol in water for 6 h at 80ºC. After filtration and evaporation of the extracted solution, the residue (240 g) was partitioned between *n*-butanol and water. The *n*-butanol layer was evaporated and lyophilized yielding light brown powder (18 g).

Primary cultures of rat cortical cells

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 as previously described (Cho *et al.*, 2000). Proliferation of non-neuronal cells was arrested by the addition of 10 μM cytosine arabinoside at 7 days after plating (Cho *et al.*, 2000). All experiments were performed at 10-12 days after plating.

Induction of neuronal damage and treatment of cells with SK OFB901

Oxidative neuronal damage was induced by the exposure of cultured cells to H_2O_2 (100 μM) for 5 min or xanthine (0.5 mM)/xanthine oxidase (10 mU/ml) for 10 min in HEPES-buffered salt solution (HBSS) as described (Dok-Go *et al.*, 2003). Excitotoxic damage was induced by the exposure to L-glutamate (100 μM) or NMDA (100 μM) for 15 min in Mg^{2+}-free HBSS as described (Cho *et al.*, 2001). After the exposures to oxidative stress or excitotoxins, cultures were washed three times with HBSS and maintained at 37ºC for 20-24 h in MEM supplemented with 21 mM glucose. KA-induced toxicity or A_β toxicity was respectively induced by the exposure to KA (50 μM) or $A_{\beta(25-35)}$ (40 μM) for 24 h in MEM supplemented with 21 mM glucose as described (Yoon *et al.*, 2004; Cho *et al.*, 2005).

To evaluate the effects of SK OFB901 on the neuronal cell damage induced as described above, the cultures were simultaneously exposed to various concentrations of SK OFB901 during the respective insults. The stock solution of SK OFB901 was prepared in 100% dimethyl sulfoxide (DMSO) at 200-fold the highest concentration tested and then serially diluted to the desired concentrations. For control treatment, sister cultures were exposed to 0.5% DMSO, which showed no effects on cell viability (Cho *et al.*, 2000).

Assessment of cell viability

Following the treatment of cells, cell viability was assessed using MTT reduction assays (Hansen *et al.*, 1989; Cho *et al.*, 2005). In brief, MTT was added to each well at the final concentration of 0.5 mg/ml, and the cells were incubated at 37ºC for 3 h. The media were then removed and the formazan crystals produced in the wells were dissolved by the addition of DMSO. The absorbance was measured at 550 nm using a $VERSA_{max}$ microplate reader (Molecular Devices, USA). The percent cell survival was calculated using the following formula:

$$\text{Cell survival (\%)} = 100 \times (Abs_{insult+sample} - Abs_{insult})/(Abs_{control} - Abs_{insult})$$

Assay of lipid peroxidation in rat brain homogenates

Lipid peroxidation was initiated by Fe^{2+} (10 μM) and L-ascorbic acid (100 μM) in rat forebrain homogenates, and assayed as previously described (Cho and Lee, 2004). In brief, the reaction mixture was incubated at 37ºC for 1 h in the absence or presence of various concentrations of SK OFB901. The reaction was stopped by the addition of trichloroacetic acid (28% w/v) and thiobarbituric acid (1% w/v) in succession, and the mixture was then heated at 100ºC for 15 min. After centrifugation to remove precipitates, the absorbance was measured at 532 nm. The percent inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = 100 \times (Abs_{control} - Abs_{sample})/Abs_{control}$$

Assay for DPPH radical scavenging activity

DPPH radical scavenging activity was measured according to the method previously described (Cho and Lee, 2004). Briefly, the reaction mixture containing various concentrations of SK OFB901 and DPPH methanolic solution (150 μM) was incubated at 37ºC for 30 min and the absorbance was measured at 520 nm. The percent scavenging activity was calculated using the above formula.

Data calculation

All experiments were performed at least three times in duplicate. Data are expressed as means±S.E.M. IC_{50} values, 50% inhibitory concentrations, were determined by non-linear regression of the mean values using Prism (GraphPad Software Inc., USA). Statistical analysis was performed by Student's *t*-test with a *p* value of less than 0.05 being considered statistically significant.

RESULTS

Inhibition of oxidative stress-induced neuronal damage by SK OFB901

To evaluate the effects on various types of oxidative neuronal damage, cultured cells were exposed to either H_2O_2 or xanthine/xanthine oxidase in the presence of various concentrations of SK OFB901. In consistence with our previous findings (Dok-Go *et al.*, 2003; Cho and Lee, 2004), approximately 80-90% of the cells were damaged at 20-24 h after the exposure to H_2O_2 or xanthine/xanthine oxidase. The reduced cell viability was dramatically and concentration-dependently reversed by SK OFB901 (Fig. 1), demonstrating potent inhibition of the oxidative neuronal damage. The calculated IC_{50} values were 94.8 and 61.5 μg/ml, respectively.

Inhibition of excitotoxic neuronal damage by SK OFB901

We next examined the effects of SK OFB901 on the excitotoxic neuronal damage induced by glutamate, NMDA or KA in primary cultured cortical cells. In agreement with the previous reports (Choi *et al.*, 1987; Cho and Lee, 2004), the exposure of the cultured cells to excitotoxins produced marked acute neuronal swelling and resulted in the reduction of cell viability due

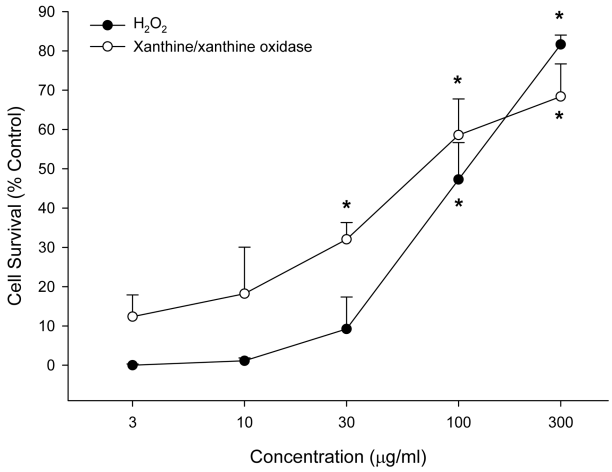

Fig. 1. Effects of SK OFB901 on the oxidative neuronal damage in primary cultured rat cortical cells. The cultured cells (10-12 days *in vitro*) were exposed to H_2O_2 (100 μM) for 5 min or xanthine (0.5 mM)/xanthine oxidase (10 mU/ml) for 10 min in the absence or presence of the indicated concentrations of SK OFB901, and cell viability was assessed using the MTT reduction assay after incubation for 20-24 h at 37ºC. Each point represents the mean±S.E.M. from at least three different experiments performed in duplicate. *, $p<0.05$ *vs* the viability of the cells exposed to the respective oxidative insults in the absence of SK OFB901.

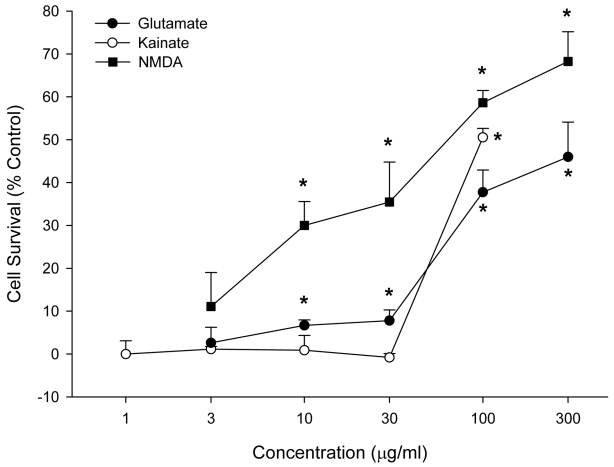

Fig. 2. Effects of SK OFB901 on the excitotoxic neuronal damage in primary cultured rat cortical cells. The cultured cells (10-12 days *in vitro*) were exposed to 100 μM glutamate or NMDA for 15 min or 50 μM KA for 24 h in the absence or presence of the indicated concentrations of SK OFB901, and cell viability was assessed using the MTT reduction assay after incubation for 20-24 h at 37°C. Each point represents the mean ± S.E.M. from at least three different experiments performed in duplicate. *, $p < 0.05$ *vs* the viability of the cells exposed to the respective excitotoxic insults in the absence of SK OFB901.

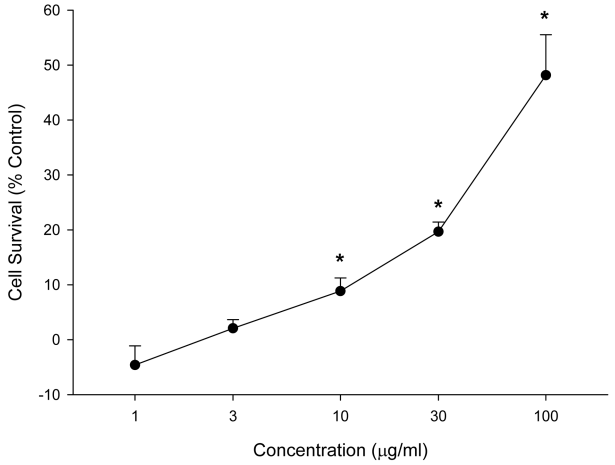

Fig. 3. Effects of SK OFB901 on the $A_{\beta(25-35)}$-induced neuronal damage in primary cultured rat cortical cells. The cultured cells (10-12 days *in vitro*) were exposed to 40 μM $A_{\beta(25-35)}$ for 24 h in the absence or presence of the indicated concentrations of SK OFB901, and cell viability was assessed using the MTT reduction assay. Each point represents the mean ± S.E.M. from three different experiments performed in duplicate. *, $p < 0.05$ *vs* the viability of the cells exposed to $A_{\beta(25-35)}$ in the absence of SK OFB901.

to the delayed cell death over 20-24 h. We found that the glutamate-induced excitotoxicity was moderately attenuated by SK OFB901 (Fig. 2). The maximal inhibition was approximately 50% at the highest concentration tested (300 μg/ml). The inhibition of the NMDA-induced toxicity by SK OFB901 was more potent (Fig. 2), with the IC_{50} value of 50.6 μg/ml. The KA-induced toxicity was also inhibited by SK OFB901, but the inhibition was less potent than that of the NMDA-induced toxicity (Fig. 2).

Inhibition of $A_{\beta(25-35)}$-induced neuronal damage by SK OFB901

In order to characterize additional pharmacological actions of *Opuntia ficus-indica* var. *saboten* in the central nervous system (CNS), we then examined the effect of SK OFB901 on the neuronal damage induced by $A_{\beta(25-35)}$, an active neurotoxic peptide fragment of A_β (Yankner *et al.*, 1990). In consistence with our previous observation (Yoon *et al.*, 2004), the exposure of the cultured cells to 40 μM $A_{\beta(25-35)}$ for 24 h decreased the cell viability to 40-60%. The $A_{\beta(25-35)}$-induced neurotoxicity was considerably inhibited by SK OFB901 (Fig. 3). Significant inhibition was observed at the concentrations of 10 μg/ml and above, with the maximal inhibition of approximately 50% at 100 μg/ml.

Inhibition of lipid peroxidation by SK OFB901 and its DPPH radical scavenging activity

We finally examined antioxidant and radical scavenging activities of SK OFB901 using cell-free bioassays. As shown in Fig. 4, SK OFB901 was shown to potently scavenge DPPH free radicals. Fifty percent scavenging activity was achieved at the concentration of 66.9 μg/ml. In addition, it dramatically inhibited lipid peroxidation initiated by Fe^{2+} and L-ascorbic acid in rat brain homogenates (Fig. 5), with the IC_{50} value of 50.0 μg/ml.

DISCUSSION

The results presented in this study confirmed and extended neuroprotective and antioxidant effects of the stems of *Opuntia ficus-indica* var. *saboten*. The butanol fraction (SK OFB901) prepared from 50% ethanol extract of the stems was shown to exert antioxidative neuroprotection, inhibiting lipid peroxidation in rat brain homogenates, scavenging DPPH radicals, and protecting cultured cells against oxidative neuronal damage. Moreover, SK OFB901 was also shown to exhibit protective actions against neuronal injuries induced by excitotoxins and A_β, implying multiple action mechanisms by which SK OFB901 exerts neuroprotective effects.

Given the suggested roles of oxidative stress and free radi-

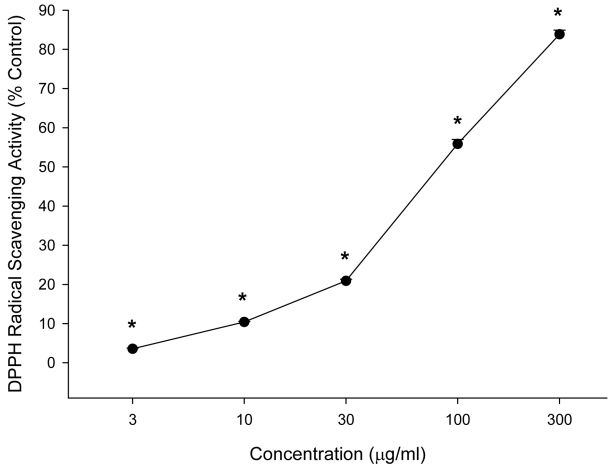

Fig. 4. DPPH radical scavenging activity of SK OFB901. DPPH radical scavenging activity was determined in the absence or presence of the indicated concentrations of SK OFB901 as described in Materials and methods. Each point represents the mean ± S.E.M. from three separate measurements performed in duplicate. *, $p < 0.05$ *vs* control.

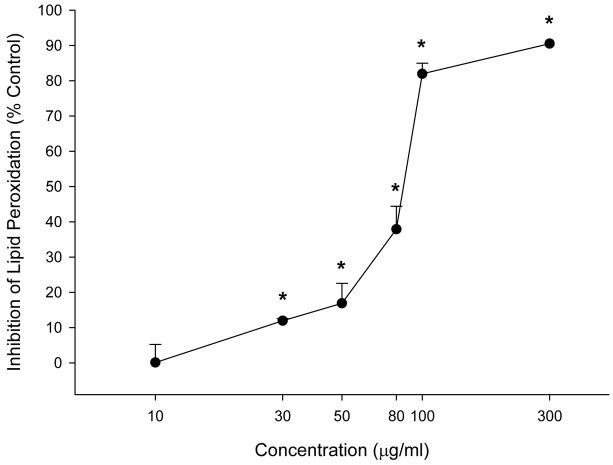

Fig. 5. Inhibition of lipid peroxidation in rat brain homogenates by SK OFB901. Lipid peroxidation initiated in rat brain homogenates by Fe^{2+} and L-ascorbic acid was assessed in the absence or presence of the indicated concentrations of SK OFB901 as described in Materials and methods. Each point represents the mean ± S.E.M. from three separate measurements performed in duplicate. *, $p < 0.05$ *vs* control.

cals in neuronal death after brain ischemia or trauma and in many other neurodegenerative disorders (Halliwell, 1992), we first examined the effects of SK OFB901 on the oxidative neuronal damage using primary cultured cortical cells. As shown in Fig. 1, the oxidative damage induced by H_2O_2 or xanthine/xanthine oxidase was dramatically and concentration-dependently inhibited by SK OFB901. Previously, we have observed that the methanol extract of the stems mildly inhibits oxidative neuronal damage in the cultured cells (Dok-Go *et al.*, 2003). Based on the measurements of lactate dehydrogenase (LDH) activity released into the culture media, the methanol extract of the stems inhibited the H_2O_2-induced oxidative damage with the IC_{50} value of 457.6 μg/ml (Dok-Go *et al.*, 2003, data not shown). The IC_{50} value of SK OFB901 determined by MTT reduction assay in this study was 94.8 μg/ml. According to our and other previous reports (Xie *et al.*, 2001; Cho *et al.*, 2005), the cell viability assessed by LDH assay and MTT assay is believed to exhibit comparable results. Thus, SK OFB901 appears to exert approximately 5-fold more potent inhibition of the oxidative neuronal damage than the methanol extract of the stems.

The exposure of cultured cells to H_2O_2 or xanthine/xanthine oxidase generates free radicals such as hydroxyl and superoxide radicals, which are believed to actively participate in the initiation of lipid peroxidation and eventually cause cell death by damaging all types of biomolecules, including proteins, lipids, and DNA (Halliwell, 1992). Thus, we next examined if SK OFB901 scavenges free radicals, and if it inhibits lipid peroxidation. As shown in Figs. 4 and 5, SK OFB901 exhibited strong antioxidant properties, scavenging DPPH free radicals and inhibiting lipid peroxidation induced in rat brain homogenates by Fe^{2+} and L-ascorbic acid. Moreover, SK OFB901 was recently reported to inhibit NO production in LPS-activated microglia and scavenge peroxynitrite (Lee *et al.*, 2006). The peroxynitrite is a toxic mediator in inflammatory processes with strong oxidizing properties toward many biomolecules (Darley-Usmar and Halliwell, 1996). In the presence of NO, peroxynitrite would be formed in combination with superoxide anion and other oxidants, and subsequently cause neuronal damage (Darley-Usmar and Halliwell, 1996; Wang *et al.*, 2003; Lee *et al.*, 2006). Brain tissue is known to be particularly susceptible to oxidative damage (Reiter, 1995). Thus, the antioxidant effect with radical scavenging activities of SK OFB901 as well as its protective action against the oxidative neuronal damage may be beneficial for the oxidative stress-associated brain disorders.

L-glutamate is the principal excitatory amino acid neurotransmitter in the mammalian CNS. Accumulating evidence indicates that excessive release of glutamate is also known to be a major cause for neuronal damage in many neurodegenerative disorders (Sauer and Fagg, 1992). Overstimulation of glutamatergic receptors provokes neurotoxic mechanisms including the production of reactive oxygen species, eventually resulting in cell death by oxidizing cellular components (Simo-

nian and Coyle, 1996). In this study, we found that SK OFB901 protected cortical cells against the excitotoxicity induced by glutamate, NMDA or KA. As shown in Fig. 2, the NMDA-induced toxicity was inhibited more potently and dramatically, with the IC_{50} of 50.6 μg/ml. According to the recent paper reported by Kim *et al.* (2006), the methanol extract of the fruits was also capable of inhibiting NMDA-, KA-, or oxygen-glucose deprivation-induced neurotoxicity. They observed that pre- and co-treated mouse cortical cells with the fruit methanol extract at the concentrations of 300 μg/ml and above protected from the toxicity induced by NMDA (25 μM) for 20-24 h (Fig. 1 in Kim *et al.*, 2006). Although it may be difficult to directly compare their data to ours because of the different experimental systems and conditions employed, our butanol fraction from the stems seems to exert more potent inhibition of the NMDA toxicity (Fig. 2) than the fruit methanol extract. Similarly, the KA toxicity appears to be inhibited more potently by SK OFB901 than by the fruit methanol extract (Fig. 2 in this study *vs* Fig. 1 in Kim *et al.*, 2006).

To further expand additional pharmacological actions of SK OFB901, we then examined its effects on the neuronal damage induced by $A_{\beta(25-35)}$, the neurotoxic fragment of the A_β peptides that are believed to be one of the major factors in Alzheimer's disease (Selkoe and Schenk, 2003). The $A_{\beta(25-35)}$-induced neurotoxicity was significantly reduced by SK OFB901 (Fig. 3). The A_β toxicity is suggested to be associated with increases in reactive oxygen species, which may in turn initiate neurotoxic events (Behl and Moosmann, 2002). Therefore, it is possible that the antioxidant properties with radical scavenging activities of SK OFB901 may contribute, at least in part, to its protective action against the A_β toxicity.

Many constituents including various flavonoids have been isolated from the stems (Lee *et al.*, 2003; Saleem *et al.*, 2006). We previously proposed that, among the isolated constituents, quercetin, (+)-dihydroquercetin, and quercetin 3-methyl ether are the active antioxidant principles exhibiting neuroprotective actions against the oxidative neuronal injuries (Dok-Go *et al.*, 2003). Further studies are currently under investigation to identify active principle(s) in SK OFB901.

In conclusion, the present study demonstrated that SK OFB901, the butanol fraction prepared from the stems of *Opuntia ficus-indica* var. *saboten*, exerts strong antioxidant and neuroprotective effects through multiple mechanisms. Our findings presented in this study expanded pharmacological profiles of *Opuntia* stems by demonstrating protective actions of SK OFB901 against the A_β toxicity as well as the oxidative neuronal damage or excitotocixity. The inhibition of the H_2O_2-induced oxidative damage and excitotoxicity by SK OFB901 appears to be more potent than by the methanol extracts of the fruits or stems. These findings imply that SK OFB901 could serve as an excellent candidate for the development of health-promoting preparations that may be beneficial for the prevention or management of neurodegenerative disorders associated with oxidative stress, excitotoxicity, and A_β.

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