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# Gossypin Protects Primary Cultured Rat Cortical Cells from Oxidative Stress- and β-Amyloid-Induced Toxicity

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The present study investigated the effects of gossypin, 3,3',4',5,7,8-hexahydroxyflavone 8-glucoside, on the toxicity induced by oxidative stress or β-amyloid ($A_\beta$) in primary cultured rat cortical cells. The antioxidant properties of gossypin were also evaluated by cell-free assays. Gossypin was found to inhibit the oxidative neuronal damage induced by xanthine/xanthine oxidase or by a glutathione depleting agent, D,L-buthionine (*S,R*)-sulfoximine. In addition, gossypin significantly attenuated the neurotoxicity induced by $A_{\beta(25-35)}$. Furthermore, gossypin dramatically inhibited lipid peroxidation initiated by $Fe^{2+}$ and ascorbic acid in rat brain homogenates. It also exhibited potent radical scavenging activity generated from 1,1-diphenyl-2-picrylhydrazyl. These results indicate that gossypin exerts neuroprotective effects in the cultured cortical cells by inhibiting oxidative stress- and $A_\beta$-induced toxicity, and that the antioxidant properties of gossypin may contribute to its neuroprotective actions.

**Key words:** Gossypin, Neuroprotection, Oxidative damage, Cortical neurons, Beta-amyloid, Antioxidant

## INTRODUCTION

Oxidative stress refers to the undue oxidation of biomolecules, such as membrane lipids, proteins and DNA. It is carried out by reactive oxygen species (ROS), ultimately leading to cellular damage and subsequent cell death (Simonian and Coyle, 1996; Behl and Moosmann, 2002). Brain tissue is known to be particularly susceptible to oxidative damage due to its high utilization of oxygen, high contents of unsaturated lipids and relatively deficient antioxidant defense mechanisms (Reiter, 1995). Thus, oxidative brain damage induced by various oxidative stresses has been implicated in the pathogenesis of a number of acute and chronic neurodegenerative disorders including Alzheimer's disease (AD) (Halliwell, 1992; Behl and Moosmann, 2002).

Other lines of evidence indicate that one of the major factors causing neurodegenerative processes in AD is believed to be β-amyloid ($A_\beta$) peptides. Although the precise mechanisms mediating $A_\beta$ toxicity remain to be fully elucidated, $A_\beta$ toxicity is suggested to be associated with increases in ROS, which may in turn initiate neurotoxic events (Behl *et al.*, 1994; Harris *et al.*, 1995; Smith *et al.*, 1996). Furthermore, it has been proposed that the increased ROS and free radicals would generate more $A_\beta$, which in turn would cause more oxidative stress and exacerbate the toxicity through a vicious cycle (Dyrks *et al.*, 1992; Hensley *et al.*, 1994; Zhang *et al.*, 1997). These findings strongly support the hypothesis that oxidative stress plays a pivotal role in $A_\beta$ toxicity. Therefore, pharmacological agents acting to protect neurons from the toxicity induced by oxidative stress or $A_\beta$ may provide useful therapeutic potential for AD.

Flavonoids are a group of polyphenolic compounds found ubiquitously in plants. They exhibit a variety of biological activities, such as antiinflammatory, antioxidant, antiviral and antitumor actions (Formica and Regelson, 1995). Gossypin (3,3',4',5,7,8-hexahydroxyflavone 8-glucoside, Fig. 1), a flavonoid known to be present in Hibiscus spp., has been reported to exhibit antiinflammatory action through the inhibition of arachidonic acid metabolism (Ferrandiz and Alcaraz, 1991). It has also been shown to inhibit acetic acid-induced writhing in mice (Viswanathan

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*et al.*, 1984). The analgesic effect was antagonized by naloxone, indicating opioid receptor-mediated action. Later, Viswanathan *et al.* (1993) suggested that multi-neurotransmitter pathways, including cholinergic and GABA receptors, are involved in gossypin-induced anti-nociception.

To investigate the pharmacological actions of gossypin in the central nervous system, primary cultured rat cortical cells were employed in the present study. Effects of gossypin were examined on the neuronal damage induced in the cultured cells by oxidative stress or $A_{\beta(25-35)}$. The antioxidant properties of gossypin were also evaluated in this study by cell-free *in vitro* assays.

## MATERIALS AND METHODS

### Materials

Materials used for cell cultures, including minimum essential medium (MEM, with Earle's salts), fetal calf serum and horse serum, were obtained from Gibco BRL (Gaithersburg, USA). Gossypin (from *Hibiscus vitifolius* flowers, FW = 648.5, purity > 90%), laminin, xanthine (X), xanthine oxidase (XO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), D,L-buthionine (*S,R*)-sulfoximine (BSO) and lactate dehydrogenase (LDH) assay kit were purchased from Sigma (St. Louis, USA). $A_{\beta(25-35)}$ was obtained from Bachem (Merseyside, UK). All other chemicals were reagent grade or better.

Timed-pregnant Sprague-Dawley (SD) rats for primary cortical cell cultures and male SD rats for preparations of brain homogenates were obtained from Daehan Biolink (Chungbuk, Korea). The animals were maintained in our animal facility, with Purina laboratory chow and water *ad libitum,* with a 12 h light cycle at a controlled temperature (22±2°C) until utilized.

### 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.*, 2001). In brief, cortices were dissected and mechanically dissociated into single cells by triturations through fire-polished Pasteur pipettes. Cells were plated at a density of $4\text{-}5\times10^5$ cells per well on 24-well culture plates coated with poly-L-lysine and laminin. Cultures were incubated at 37°C in a humidified atmosphere of 95% air/5% $CO_2$, in a medium consisting of MEM supplemented with glucose (final concentration, 25 mM), glutamine (2 mM), fetal calf serum (5%) and horse serum (5%). Proliferation of non-neuronal cells was arrested by the addition of 10 μM cytosine arabinoside at 7 days after plating (Cho *et al.*, 2000; 2001).

### Experimental treatments and the assessment of cell damage

Oxidative damage was induced at 10-13 days after plating by the exposure of the cultures to X(0.5 mM)/XO (10 mU/mL) for 10 min in HEPES-buffered salt solution, as described (Jung *et al.*, 2002). After the exposure, cultures were washed and maintained for 20-24 h in MEM supplemented with glucose, and then the extent of cell damage was quantified by measuring LDH activity released into the culture media (Jung *et al.*, 2002; Cho *et al.*, 2002). The BSO-induced oxidative damage or the $A_\beta$-induced toxicity was respectively induced by the exposure of cultures to 1 mM BSO or 40 μM $A_{\beta(25-35)}$ for 24 h in MEM supplemented with glucose (Cho and Lee, 2004). Cell damage was then assessed by LDH assays.

To evaluate the effects of gossypin on the toxicity induced by X/XO, BSO, or $A_{\beta(25-35)}$, cultures were simultaneously exposed to various concentrations of gossypin during the respective insults. Stock solution of gossypin was prepared in 100% dimethylsulfoxide (DMSO) at 200x the highest concentration tested and then serially diluted to the desired concentrations. For control treatment, sister cultures were exposed to 0.5% DMSO, which was shown to exhibit no effect on cell viability (Cho *et al.*, 2000).

### Assay of lipid peroxidation in the rat brain homogenates

Lipid peroxidation was initiated by 10 μM $Fe^{+2}$ and 100 μM ascorbic acid in the rat brain homogenates, and assayed as previously described (Cho and Lee, 2004). Briefly, the reaction mixture was incubated at 37°C for 1 h in the presence of various concentrations of gossypin. 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 100°C for 15 min. After centrifugation to remove precipitates, absorbance was measured at 532 nm using a $VERSA_{max}$ microplate reader (Molecular Devices, Sunnyvale, USA). The percentage of 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). In brief, the reaction mixture containing various concentrations of gossypin and 150 μM DPPH methanolic solution was incubated at 37°C for 30 min and absorbance was measured at 520 nm. The percentage of scavenging activity was calculated using the above formula.

### Data calculation

$IC_{50}$ values, concentrations exhibiting 50% inhibition, were determined by non-linear regression using Prism

(GraphPad Software Inc., USA).

## RESULTS

### Inhibition of oxidative stress-induced toxicity by gossypin

To evaluate the neuroprotective effects of gossypin (Fig. 1), primary cultures of rat cortical cells containing neuronal and non-neuronal cells maintained for 10-13 days *in vitro* were utilized. Consistent with our previous reports (Jung *et al.*, 2002; Dok-Go *et al.*, 2003), prominent oxidative cell damage (Fig. 2B) was observed under a phase-contrast microscope at 20-24 h after the exposure to X/XO for 10 min. Based on LDH measurements in the culture media, approximately 80-90% cells were found to be damaged (Jung *et al.*, 2002; Dok-Go *et al.*, 2003; Cho and Lee, 2004). The X/XO-induced oxidative cell damage was reduced in the presence of gossypin at 30 μg/mL (Fig. 2C). As shown in Fig. 2D, the inhibition was concentration-dependent, and the calculated $IC_{50}$ was 44.9 μg/mL.

Oxidative damage was then induced by exposing cultures to BSO, a GSH depleting agent (Griffith and Meister, 1979). Based on LDH assays, approximately 50% of cells were damaged after 24 h of exposure to 1 mM BSO. Simultaneous treatments with gossypin during the BSO exposures dramatically and potently inhibited the BSO-induced toxicity (Fig. 3). The $IC_{50}$ was 7.4 μg/mL.

### Attenuation of $A_{\beta(25-35)}$-induced neurotoxicity by gossypin

Next, the effect of gossypin on the toxicity induced by $A_{\beta(25-35)}$ was examined in cultured cells. Based on LDH assays, a 24 h exposure of rat cortical cells to 40 μM $A_{\beta(25-35)}$ produced approximately 45-60% cell damage. As illustrated in Fig. 4, gossypin attenuated the $A_{\beta(25-35)}$-induced toxicity in a concentration-dependent fashion. The calculated $IC_{50}$ was 155.6 μg/mL.

### Inhibition of lipid peroxidation by gossypin and its radical scavenging activity

Finally, the antioxidative properties of gossypin were

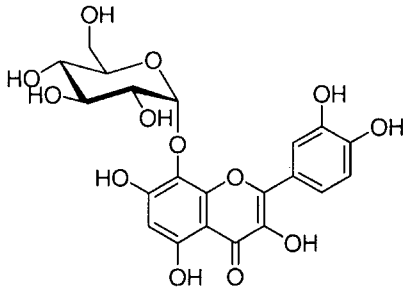


**Fig. 1.** The structure of gossypin (3,3',4',5,7,8-hexahydroxyflavone 8-glucoside)

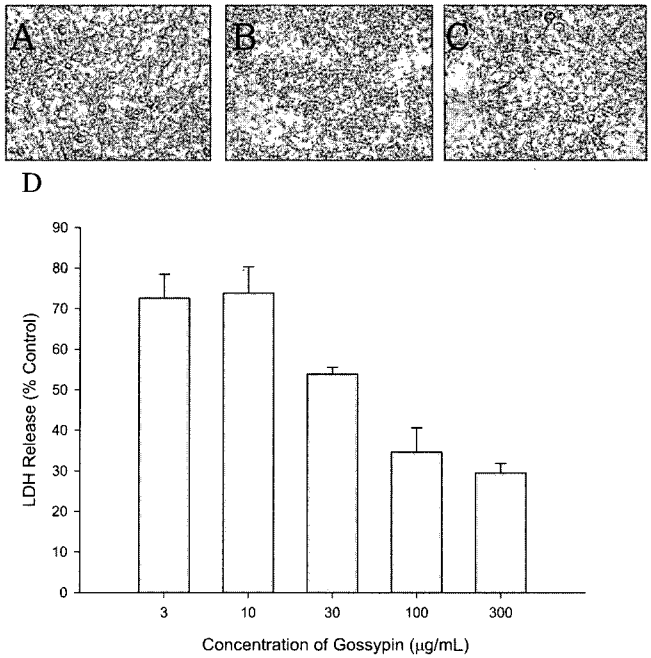


**Fig. 2.** Inhibition of the xanthine (X)/xanthine oxidase (XO)-induced oxidative damage by gossypin. Phase-contrast photomicrographs of primary cultured rat cortical cells (12 days after plating) are shown (A) at 24 h after the exposure to X/XO (0.5 mM/10 mU/mL) for 10 min in the absence (B) or presence of gossypin at 30 μg/mL (C). LDH activities in the culture media exposed to X/XO in the absence or presence of the indicated concentrations of gossypin were measured after 20-24 h of exposure (D). Data were calculated as percentages of control LDH activity measured in the absence of gossypin. Each point represents the mean ± SEM from at least three measurements performed in duplicate. (Magnification, 200x).

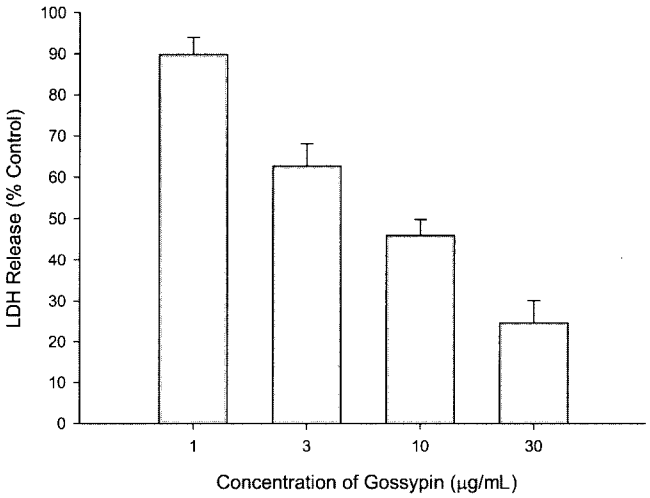


**Fig. 3.** Inhibition of the D,L-buthionine (*S,R*)-sulfoximine (BSO)-induced oxidative damage by gossypin. Cortical cultures (10-13 days *in vitro*) were exposed to 1 mM BSO in the absence or presence of the indicated concentrations of gossypin. LDH activities in the culture media were measured after 24 h of exposure. Each point represents the mean ± SEM from at least three measurements performed in duplicate.

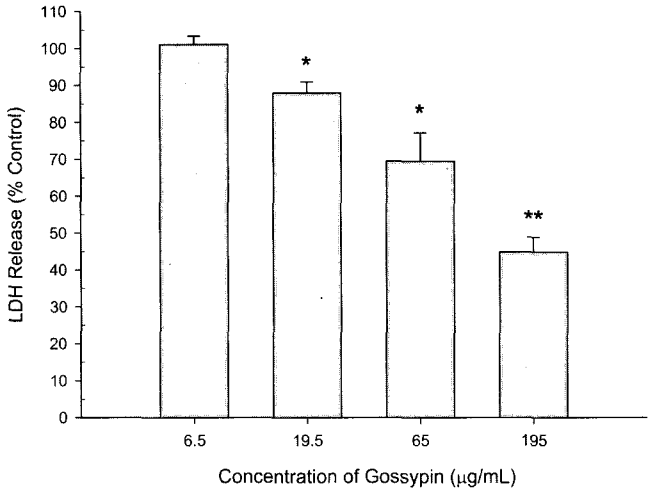


**Fig. 4.** Attenuation of the $A_{\beta(25-35)}$-induced neurotoxicity by gossypin. Cortical cultures (10-13 days *in vitro*) were exposed to 40 μM $A_{\beta(25-35)}$ in the absence or presence of the indicated concentrations of gossypin. LDH activities in the culture media were measured after 24 h of exposure. Each point represents the mean ± SEM from at least three measurements performed in duplicate. (*$p < 0.05$; **$p < 0.01$ by paired t-test).

evaluated by cell-free *in vitro* assays. Gossypin exhibited dramatic inhibition of lipid peroxidation initiated by $Fe^{2+}$ and ascorbic acid in rat brain homogenates (Fig. 5A). It also exhibited potent DPPH radical scavenging activity (Fig. 5B). The respective $IC_{50}$ values were 7.3 and 6.2 μg/mL.

## DISCUSSION

Many, but not all flavonoids, were shown to exhibit neuroprotective effects in different experimental systems. For example, quercetin, luteolin and fisetin were reported to be effective in protection against oxidative stress induced by glutamate in hippocampal cell line HT-22 cells, whereas apigenin and chrysin were not effective (Ishige *et al.*, 2001; Behl and Moosmann, 2002). Recently, it has been shown in primary cultured cortical cells that quercetin, (+)-dihydroquercetin, and quercetin 3-methyl ether, the flavonoids isolated from *Opuntia ficus-indica* var. *saboten*, protect from oxidative damage induced by X/XO or $H_2O_2$ (Dok-Go *et al.*, 2003). In addition, wogonin, a flavonoid present in *Scutellaria baicalensis* Georgi, was reported to inhibit the X/XO-, $H_2O_2$-, or BSO-induced oxidative damage and the glutamate- or NMDA-induced excitotoxicity in cortical cultures (Cho and Lee, 2004). This study reports the neuroprotective and antioxidant effects of gossypin, a flavonoid obtained from *Hibiscus vitifolius* flowers.

The exposure of cultured cells to X/XO generates superoxide radicals, which are believed to actively participate in the initiation of lipid peroxidation and eventually

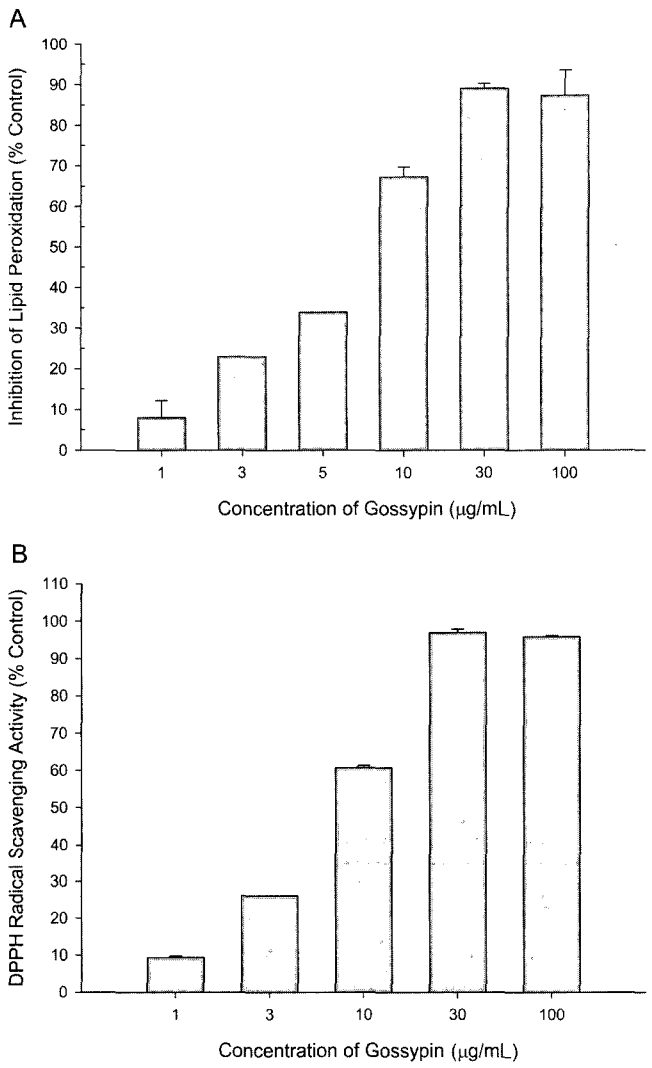


**Fig. 5.** Effects of gossypin on lipid peroxidation and DPPH radicals. (A) Lipid peroxidation initiated by $Fe^{+2}$ and ascorbic acid in brain homogenates was assessed as described in the Materials and Methods section, in the absence or presence of the indicated concentrations of gossypin. (B) DPPH radical scavenging activity was determined in the absence or presence of the indicated concentrations of gossypin as described in the Materials and Methods section. Each point represents the mean ± SEM from three measurements performed in duplicate.

cause cell death (Halliwell, 1992; Simonian and Coyle, 1996; Dok-Go *et al.*, 2003). In this study, it was found that gossypin inhibited the X/XO-induced oxidative damage in cortical cultures (Fig. 2). This finding is the first demonstration of the neuroprotective action exerted by gossypin. At this point, however, it is not clear whether gossypin inhibits the X/XO-induced oxidative damage by blocking the generation of superoxide radicals, or by acting directly on radicals as a scavenger, or both. Measurements of superoxide radicals in gossypin-treated cells would help to elucidate the exact action mechanism.

Depletion of GSH is believed to be associated with

neurodegenerative disorders including Parkinson's disease and AD (Bains and Shaw, 1997). It can enhance oxidative stress and may also increase the levels of excitotoxic molecules. GSH depletion is experimentally induced in cultured cells by blocking GSH synthesis with BSO, an inhibitor of γ-glutamylcysteine synthetase (Griffith and Meister, 1979). Li *et al.* (1997) showed that BSO treatment of cortical neurons and neuronal cell lines lowered GSH levels, and ultimately caused cell death. They found that activation of neuronal 12-lipoxygenase is involved in the BSO-induced toxicity, and that 12-lipoxygenase inhibitors, such as baicalein, inhibit the toxicity (Li *et al.*, 1997). Exposure of the cultured cells to BSO produced approximately 50% cell damage, as assessed by LDH activity in the culture medium. It was found that gossypin markedly inhibited the BSO-induced damage (Fig. 3), with an $IC_{50}$ of 7.4 μg/mL. Together, these results indicate that gossypin protects cortical cells from oxidative neuronal damage induced by not only X/XO but also BSO. Gossypin inhibited the BSO-induced oxidative damage more potently than the X/XO-induced damage.

One of the early events induced by $A_\beta$ in the AD brain could be GSH depletion. Since $A_\beta$ has previously been shown to induce GSH depletion in cultured neurons (Muller *et al.*, 1997; Bains and Shaw, 1997), and since it has been shown in this report that gossypin inhibits GSH depletion-induced cell damage (Fig. 3), we next examined if gossypin affects $A_\beta$-induced neurotoxicity. Treatment of cultured cortical cells with $A_{\beta(25\text{-}35)}$, an active fragment of $A_\beta$ peptide, produced 45-60% neuronal death. It was found that the $A_{\beta(25\text{-}35)}$-induced toxicity was significantly attenuated by gossypin (Fig. 4). Although approximately 150 μg/mL of gossypin is required to achieve 50% inhibition, which is quite a high concentration compared to the $IC_{50}$ values inhibiting either X/XO- or BSO-induced toxicity, the effect has been shown to be statistically significant at 20 μg/mL and above (Fig. 4). Harkany *et al.* (1999) proposed that the $A_\beta$ toxicity is mediated by multiple signaling pathways. The presence of multiple signaling pathways may explain, at least in part, why the effect of gossypin on the $A_\beta$ toxicity is not as dramatic as its effects on the oxidative damage.

These findings, showing that gossypin inhibits oxidative stress- and $A_{\beta(25\text{-}35)}$-induced toxicity, strongly imply that it may act as an antioxidant. Indeed, gossypin was found in this study to potently inhibit lipid peroxidation (Fig. 5A) and scavenge DPPH radicals (Fig. 5B). Thus, like many other flavonoids, gossypin exhibits antioxidant actions with radical scavenging activity, which may contribute to the neuroprotective effects observed in cultured cortical cells (Figs. 2-4).

We previously reported the antioxidant and neuroprotective effects of quercetin, a well-known antioxidant flavonoid (Dok-Go *et al.*, 2003). Quercetin exhibited dramatic and potent inhibition of the oxidative damage induced in cortical cultures by X/XO or $H_2O_2$ (Dok-Go *et al.*, 2003). In contrast, $H_2O_2$-induced damage was only partially inhibited by gossypin, up to 30% (data not shown). These observations imply that quercetin and gossypin may act differentially on various radicals. As pointed out previously, quercetin exhibits prooxidant action at high concentrations (Johnson and Loo, 2000; Dok-Go *et al.*, 2003). It was no longer protective against oxidative neuronal damage at concentrations of 30 μg/mL and above (Dok-Go *et al.*, 2003). However, gossypin was shown to retain protective effects on the X/XO-induced toxicity up to 300 μg/mL (Fig. 2), indicating no cytotoxic or prooxidant activity at this concentration.

Taken together, this report has demonstrated the neuroprotective and antioxidant effects of gossypin and elucidated its novel pharmacological action. Gossypin inhibited the X/XO- and BSO-induced oxidative damage and attenuated the $A_{\beta(25\text{-}35)}$-induced neurotoxicity in primary cultured rat cortical cells. In addition, gossypin inhibited lipid peroxidation in brain homogenates and scavenged DPPH radicals. The antioxidant action with radical scavenging activity may contribute to its neuroprotective effects. Based on these results, gossypin may exert beneficial actions on oxidative stress-related neurodegenerative disorders including AD. Further studies to elucidate the exact mechanisms by which gossypin exerts neuroprotective effects are currently under investigation in this laboratory.

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