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# Antiinflammatory effects of genipin, an active principle of gardenia

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**Abstract**

Genipin, the aglycone of geniposide, is metabolically produced from the geniposide in body tissues. The purpose of this study is to clarify some pharmacological actions of genipin. Genipin showed concentration-dependent inhibition on lipid peroxidation induced by $Fe^{++}$/ascorbate in rat brain homogenate. Genipin exhibited significant topical antiinflammatory effect shown as an inhibition of croton oil-induced ear edema in mice. Nitric oxide (NO) synthesis by inducible nitric oxide synthase (iNOS) is increased in inflammatory diseases and leads to cellular injury. Genipin concentration-dependently (50–300 μM) inhibited NO production and iNOS expression upon stimulation by lipopolysaccharide/interferon-γ (IFN-γ) in RAW 264.7, a murine macrophage cell line. Genipin markedly blocked lipopolysaccharide-evoked degradation of inhibitor-κB-β (IκB-β), indicating that it exhibits inhibitory effect on NO production through the inhibition of nuclear factor-κB (NF-κB) activation. It was also shown to contain potent antiangiogenic activity in a dose-dependent manner, which was detected by chick embryo chorioallantoic membrane assay. In summary, we demonstrate that genipin possesses antiinflammatory and is a specific hydroxyl radical scavenger. Its antiangiogenic and NO production-inhibitory properties are also presented.


*Keywords:* Angiogenesis; Antiinflammatory; Genipin; Inducible nitric oxide synthase; Lipid peroxidation; NO (Nitric oxide)

## 1. Introduction

The fruit of gardenia (*Gardenia jasminoides* Ellis) is an oriental folk medicine which has been included in traditional formulations. Its folkloric use is for the treatment of inflammation, jaundice, headache, edema, fever, hepatic disorders and hypertension for years (Tseng et al., 1995), and its pigments are used as food colorants in oriental countries. Its pharmacological actions such as protective activity against oxidative damage, cytotoxic effect, antiinflammatory activity and fibrolytic activity have already been elucidated (Tseng et al., 1995; Jagedeeswaran et al., 2000).

Geniposide (Fig. 1) is one of the major iridoid glycosides in the fruits of dried gardenia and is hydrolyzed to the aglycone genipin (Fig. 1) by β-D-glucosidases in the intestine and the liver (Akao et al., 1994). Recently, geniposide was identified from roots of Vietnamese *Paederia scandens* (Lour) Merrill (Quang et al., 2002) and stems of *Randia spinosa* (Hamerski et al., 2003), which are used in folk medicine. Geniposide has been reported to possess inhibitory activity on 5-lipoxygenase (Nishizawa et al., 1988), activity against tumor-promoting 12-*O*-tetradecanoylphorbol-13-acetate with an activation of protein kinase C (Lee et al., 1995) and inhibitory effects on ovalbumin-induced junction permeability and recovery of transepithelial electrical resistance in guinea pig trachea, showing its potential as an antiasthma therapy (Liaw and Chao, 2001). It was also shown to contain radioprotective activity after sublethal irradiation in mice (Hsu et al., 1997) and modulating activity on cytochrome *P*-450-dependent monooxygenase, glutathione and glutathione S-transferase in rat liver (Kang et al., 1997). However, pharmacological actions of genipin, a metabolite of geniposide, have not been well documented

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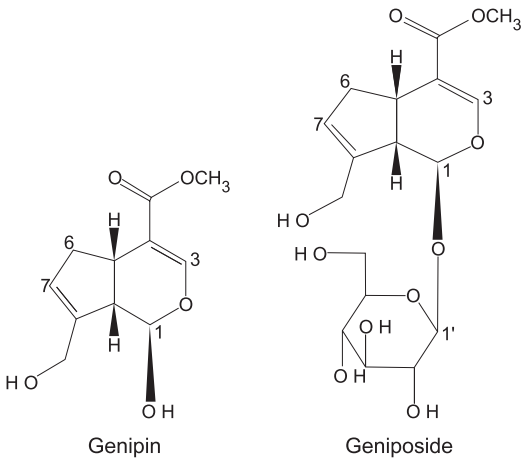


Fig. 1. The chemical structures of genipin and geniposide.

yet. Genipin has been shown to inhibit hepatocyte apoptosis induced by transforming growth factor β1 via the interference with mitochondrial permeability transition (Yamamoto et al., 2001) and protect hippocampal neurons from Alzheimer's amyloid β protein toxicity (Yamazaki et al., 2001). Although gardenia fruit has been used for the treatment of inflammation, its antiinflammatory mechanism remains to be investigated. One finding suggests that geniposide has an antiinflammatory effect (Nishizawa et al., 1988). Because geniposide is transformed into genipin by bacterial enzymes in the body (Akao et al., 1994), it may be that genipin mainly plays an important role in the efficacy. Thus, when geniposide is orally administered, genipin seems to be effectively produced in the intestine and then absorbed. In this article, we have presented some pharmacological actions of genipin.

## 2. Materials and methods

### 2.1. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco-BRL (Gaithersburg, MD). LPO-586™ for the lipid peroxidation assay was obtained from Bioxytech (Gagny, France). Bradford protein dye reagent was purchased from Bio-Rad (Melvile, NY). Genipin was purchased from Wako (Osaka, Japan). Glucose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), croton oil, ascorbic acid, indomethacin, Griess reagent, sodium nitrite, sodium dodecyl sulfate (SDS), EDTA, leupeptin, pepstatin, phenylmethanosulfonyl fluoride (PMSF), Tween 20, retinoic acid, dimethyl sulfoxide (DMSO), Trizma base, xanthine, xanthine oxidase, HEPES, $H_2O_2$, 1,1-diphenyl-2-picrylhydrazyl (DPPH), BHT (butylated hydroxytoluene), interferon-γ (IFN-γ) and lipopolysaccharide from *Escherichia coli* were purchased from Sigma (St. Louis, MI). Multi-well plates were obtained from Nalge Nunc International (Rocklide, Denmark). All other chemicals were of reagent grade or better.

### 2.2. Cell culture

RAW 264.7, a murine macrophage cell line, was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in phenol red-free DMEM containing 100 U/ml penicillin G, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum and were maintained at 37 °C in a humidified incubator containing 5% $CO_2$.

### 2.3. Animals

Male ICR mice (about 25 g) or female Sprague–Dawley rats (130–150 g) were inbred and grown in the animal room at the College of Pharmacy, Sookmyung Women's University, Seoul, Korea. The animal room was maintained at 23 ± 2 °C with a 12-h light/dark cycle. Food and tap water were supplied ad libitum. The ethical guidelines described in the NIH Guide for Care and Use of Laboratory Animals were followed throughout the experiments. The fertilized eggs used in this work were purchased from Pulmuone Food, Seoul, Korea.

### 2.4. Assay for inhibition of lipid peroxidation

For the determination of the ability to inhibit iron-dependent lipid peroxidation, rat brain homogenate (20 mg/ml) was prepared in 20 mM Tris buffer (pH 7.4) and centrifuged at 3000 × *g* for 10 min at 4 °C, and the supernatant was used for the lipid peroxidation assay. The incubation mixture in a final volume of 200 μl contained rat brain homogenate (195 μl), 100 μM $Fe^{2+}$, 400 μM ascorbic acid and various concentrations of genipin dissolved in DMSO. The resulting lipid peroxidation was evaluated by the formation of malondialdehyde (Nair et al., 1986). Malondialdehyde, the main decomposition product of peroxides derived from polyunsaturated fatty acids, was determined by chromogenic reagent *N*-methyl-2-phenylindole, which reacts with malondialdehyde to yield a stable chromophore at 45 °C for 60 min (Bioxytech; Chabrier et al., 1999). The reaction mixture was centrifuged at 15,000 × *g* for 10 min to obtain a clear supernatant, and then the absorbance was measured at 586 nm.

### 2.5. Assay for DPPH radical scavenging activity

DPPH radical scavenging activities of genipin were tested according to the method previously described (Song et al., 2003). In brief, reaction mixtures containing various concentrations of genipin dissolved in DMSO and 300 μM DPPH solution in a 96-well microtiter plate were incubated at 37 °C for 30 min, and absorbance was measured at 515 nm.

### 2.6. Assay for inhibition of xanthine oxidase activity

Xanthine oxidase catalyzes the conversion of hypoxanthine to xanthine and then uric acid as a final product in the presence of molecular oxygen to yield superoxide anions (Song et al., 2003). The oxidation of xanthine by xanthine oxidase is used to produce superoxide anions. Xanthine solution (980 μl) of 100 μM in sodium phosphate buffer, pH 7.8, with 10 μl of xanthine oxidase solution (0.04 units) and 10 μl of DMSO, was incubated for 5 min at room temperature, and the formation of uric acid was measured at 295 nm against a blank sample which did not contain the enzyme but contained 10 μl of 0.1 M phosphate buffer solution (pH 7.8) instead. Absorbance was recorded for 5 min and the tests were performed in duplicate. Various concentrations of genipin (10 μl dissolved in DMSO) were added to xanthine buffer solution (980 μl) and phosphate buffer (10 μl) as a blank test. Enzyme solution (0.04 units) was added to each 10 μl of various concentrations of genipin in 980 μl of xanthine buffer solution and treated in the same manner as the control. Inhibitory effects on xanthine oxidase activity were measured by a decrease in uric acid formation.

### 2.7. Croton oil-induced ear edema test

In order to test whether genipin possesses a plausible topical antiinflammatory property, the croton oil-induced ear edema test was performed as previously described (Olajide et al., 2000). Mice were anesthetized with ether. A total of 20 μl of an acetonic solution containing 0.4 μg of croton oil and genipin at 0.55, 1.10, 2.21 or 4.42 μmol/ear was applied to the inner surface of the right ear of each mouse. The left ear remained untreated. Control animals received only the irritant while indomethacin (1.40 μmol/ear) served as the reference. The animals were sacrificed by cervical dislocation 5 h later, and two ear plugs (6 mm in diameter) were removed from both the treated ear and the untreated ear. Thickness measurements of treated and untreated ear plugs were made with an engineer micrometer, and their weights were measured. The difference in weight and thickness between the two ear plugs was taken as a measure of edematous response.

### 2.8. Chorioallantoic membrane assay

Antiangiogenic activity was measured using chorioallantoic membrane assay as previously described (Song et al., 2003). The fertilized chicken eggs used in this study were kept in a humidified egg incubator at 37 °C. After a 3.5-day incubation, about 2 ml of albumin was aspirated from the eggs with 22-gauge needle through the small hole drilled at the narrow end of the eggs, allowing the small chorioallantoic membrane and yolk sac to drop away from the shell membrane. The shell covering the air sac was punched out and removed by forceps, and the shell membrane on the floor of the air sac was peeled away. In the 4.5-day-old chick embryo, a sample-loaded Thermanox coverslip was applied to the chorioallantoic membrane surface. The chick embryo was returned to the incubator. Two days later, an appropriate volume of a 10% fat emulsion (Intralipose, 10%) was injected using a 33-gauge needle into a 6.5-day-old embryo chorioallantois. The eggs were then observed under a microscope.

### 2.9. Assay for nitrite concentration

Accumulated nitrite ($NO_2^-$) in the media of RAW 264.7 cells grown in phenol red-free DMEM was measured using an automated colorimetric assay based on the Griess reaction (Sherman et al., 1993). Cells were plated in 12-well culture plates at a density of $8 \times 10^5$ cells and incubated with lipopolysaccharide (1 μg/ml) plus IFN-γ (10 U/ml) in the presence or absence of genipin for 24 h. The supernatants (100 μl) were reacted with a 100 l Griess reagent (6 mg/ml) at room temperature for 10 min, and then $NO_2^-$ concentration was determined by measuring the absorbance at 540 nm. The standard curve was obtained using the known concentrations of sodium nitrite.

### 2.10. Immunoblot analysis

RAW 264.7 cells were incubated with lipopolysaccharide (1 μg/ml) plus IFN-γ (10 U/ml) in the presence or absence of genipin for 24 h and then washed twice with ice-cold phosphate-buffered saline (PBS). The cells were lysed in a buffer containing 20 mM HEPES (pH 7.9), 0.1 M KCl, 0.3 M NaCl, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin. Western blotting was performed as previously described (Kim et al., 2002). The supernatant after centrifugation at 15,000 × *g* for 5 min was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane in 25 mM Tris, 20% methanol and 192 mM glycine. For immunoblotting, membranes were blocked with 5% non-fat milk dissolved in PBS containing 0.1% Tween 20 (PBST) at 4 °C overnight and then incubated at room temperature with anti-inducible nitric oxide synthase (iNOS; Transduction Laboratories, Lexington, KY), anti-IκB-α (C-21; Santa Cruz Biotechnology, Santa Cruz, CA), anti-inhibitor-κB-β (IκB-β; C-20; Santa Cruz Biotechnology), antiphospho-c-*Jun* (Ser63; Cell Signaling Technology, Beverly, MA) and anti-β-actin (Sigma-Aldrich, St. Louis, MO) antibodies for 1 h. The blots were washed and incubated with goat antimouse immunoglobulin G (IgG) conjugated to peroxidase in PBST containing 3% non-fat milk for 1 h. Blots were washed in PBST two times, incubated in chemiluminescence reagents (Supersignal® detection system) and exposed to photographic film.

### 2.11. MTT reduction assay

Cell viability was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenlytetrazolium bromide (MTT) assay

(Mosmann, 1983). Briefly, cells incubated with genipin were treated with 5 μl of MTT solution (5 mg/ml) for 3 h. The cells were then lysed with isopropyl alcohol, and the absorbance was read at 540 mm.

### 2.12. Statistical analyses

The data were analyzed for statistical significance using Student's *t*-test. *P* values less than 0.05 were considered to be significant.

## 3. Results

### 3.1. Antilipoperoxidative activity of genipin

In an assay model for assessing an ability to inhibit lipid peroxidation induced by hydroxyl radicals, genipin was evaluated for its antioxidant effect in rat brain homogenate initiated by an $Fe^{2+}$/ascorbate system. Genipin and butylated hydroxytoluene, a known inhibitor of lipid peroxidation, inhibited the generation of malondialdehyde, which reacts with *N*-methyl-2-phenylindole, in a concentration-dependent manner (Fig. 2). This indicates the significant lipid peroxidation inhibiting activity of genipin. The $IC_{50}$ values of genipin and butylated hydroxytoluene were 936.2 and 3.9 μM, respectively (Fig. 2).

The free radical scavenging activity of genipin was also tested by its ability to bleach the stable radical, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), which provided information on the reactivity of genipin with a stable free radical. Genipin could not scavenge DPPH at the used concentrations (data not shown). And, it showed no effect on xanthine oxidase activity at the concentrations used in this study (data not shown). Collectively, genipin is a specific hydroxyl radical scavenger.

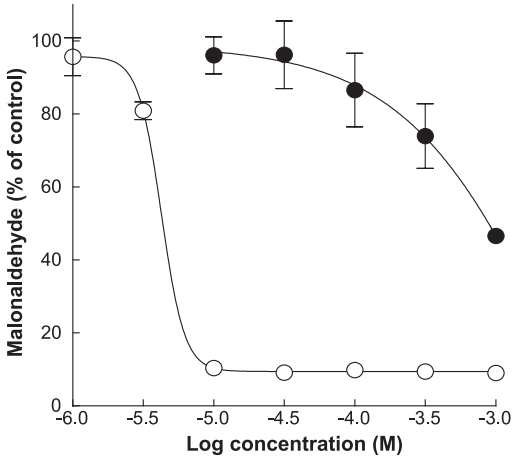


Fig. 2. Effects of different concentrations of genipin (●) and BHT (○) on lipid peroxidation product (malondialdehyde, MDA) after incubation of brain homogenate in the presence of the $FeCl_2$/ascorbic acid. Results are mean ± S.E. of three separate experiments using different brain homogenate preparations. The control value obtained from the untreated brain homogenates was measured to be 1.01 at 586 nm.

Table 1
Antiinflammatory effect of genipin on croton oil-induced ear edema in mice

| Agents | Dosages (μmol/ear) | Antiinflammatory effect[a] | |
|---|---|---|---|
| | | Edema weight (% inhibition) | Edema thickness (% inhibition) |
| Control | – | 9.32 ± 2.18 | 31.80 ± 6.87 |
| Indomethacin | 1.40 | 1.54 ± 0.98 (83.5)[b] | 6.80 ± 3.31 (78.6)[b] |
| Genipin | 0.55 | 7.30 ± 1.32 (35.5) | 27.00 ± 3.85 (15.2) |
| Genipin | 1.10 | 6.03 ± 1.11 (35.3) | 22.33 ± 3.96 (29.8) |
| Genipin | 2.21 | 5.37 ± 0.68 (42.4) | 20.50 ± 1.88 (35.6) |
| Genipin | 4.42 | 4.00 ± 1.19 (57.1)[c] | 13.67 ± 3.91 (57.1)[c] |

[a] Values represent mean ± S.E. for eight mice. Inhibition percentages in parentheses indicate relative degree of inhibition with respect to the control treated with croton oil only.
[b] $P<0.01$ compared to the control group.
[c] $P<0.05$ compared to the control group.

### 3.2. Topical antiinflammatory activity

Topical antiinflammatory activity of genipin was evaluated as the inhibition of the croton oil-induced ear edema in mice. Topical application of croton oil induced cutaneous inflammation at the ears of mice, which caused a significant increase in ear plug weight and thickness of the right ear when compared to the vehicle-treated left ear (data not shown). As a positive control, indomethacin (1.40 μmol/ear) inhibited the change in ear plug weight and thickness. Treating the mice with indomethacin significantly gave rise to an inhibition of 83.5% and 78.6% in ear plug weight and thickness, respectively (Table 1). When genipin was topically applied at 0.55, 1.10, 2.21 and 4.42 μmol/ear, it provided inhibitory effect in croton oil-induced ear edema formation in a dose-dependent fashion (Table 1). Treatment of genipin at 4.42 μmol/ear gave the same degree of inhibition (57.1%) in ear plug weight and thickness, indicating that genipin contains reasonable antiinflammatory activity.

### 3.3. Antiangiogenic activity

The chorioallantoic membrane assay was used for examining the inhibitory activity of genipin on vascular development, and retinoic acid was used as a positive control for the assay. The disk weight did not give any changes in vascular density, indicating that it could not affect the growth of blood vessels (data not shown). After the 2-day treatment, retinoic acid (1 μg/egg) gave 74.4% inhibition in the branching pattern of blood vessels (Fig. 3). When 0.125, 0.25, 0.3, 1, 3 or 10 μg/egg of genipin was applied, the inhibition percentages in the chorioallantoic membrane angiogenesis were measured to be 27.3%, 31.7%, 45.0%, 50.4%, 55.0% and 71.2%, respectively (Fig. 3). This clearly indicates that the antiangiogenic activity of genipin is dose-dependent, and its $IC_{50}$ value was calculated to be 0.74 μg/

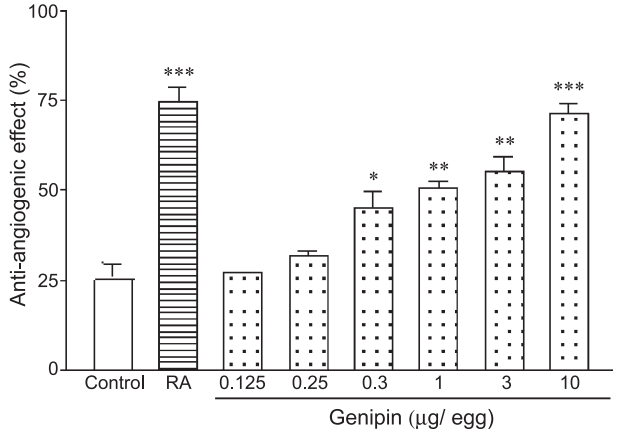


Fig. 3. Effect of genipin on chorioallantoic membrane angiogenesis. Retinoic acid (RA; 1 μg/egg) was used as a positive control. Experiments were performed in triplicate. Each column represents mean ± S.E. ***$P<0.001$; **$P<0.01$; *$P<0.05$.

egg. At a dose of 10 μg/egg, its antiangiogenic activity is comparable to that of retinoic acid (1 μg/egg) used as a positive control. Taken together, genipin significantly possesses antiangiogenic activity.

### 3.4. Effect of genipin on the lipopolysaccharide plus IFN-γ-induced iNOS expression

To assess the effect of genipin on lipopolysaccharide/IFN-γ-induced nitric oxide (NO) production in RAW 264.7 macrophages, cell culture medium was harvested, and the concentration of accumulated nitrite, the oxidative product of NO, was determined by the Griess method (Fig. 4). The accumulated nitrite, estimated by the Griess method, in the culture medium was used as an index for NO synthesis from these cells. After treatment with lipopolysaccharide plus IFN-γ for 24 h, nitrite concentration markedly increased about 13-fold ( ~ 40 μM). When cells were treated with various concentrations of genipin, nitrite production induced by lipopolysaccharide plus IFN-γ was significantly inhibited at concentrations from 50 to 300 μM in a concentration-dependent manner. No significant cytotoxic effects on cell viability were observed in these concentrations of genipin as determined by MTT reduction assay (Table 2). With the assumption that the inhibition of NO production by genipin in RAW 264.7 cells would be caused by a decrease especially in the iNOS protein, the effect of genipin on the iNOS protein expression was examined in cells treated with lipopolysaccharide plus IFN-γ for 24 h. As shown in Fig. 5, treatments with genipin inhibited iNOS protein expression induced by lipopolysaccharide plus IFN-γ in a concentration-dependent manner without changes in the amount of β-actin protein, an internal control, indicating the specific inhibition of iNOS protein expression by genipin. The results suggest that genipin contributes to the decreased iNOS protein and NO production.

Table 2
Cytotoxic effect of genipin on the viability of RAW 264.7 cells[a]

| Agents | Concentration | Relative viability[b] |
|---|---|---|
| Control | – | 100 |
| Lipopolysaccharide | 1 μg/ml | 103.5 ± 0.3 |
| Genipin | 50 μM | 103.5 ± 0.7 |
| | 70 μM | 103.8 ± 0.8 |
| | 100 μM | 104.6 ± 1.2 |
| | 300 μM | 88.5 ± 4.9 |
| | 1000 μM | 10.5 ± 1.6 |

[a] Cytotoxic effect of genipin on the viability of RAW 264.7 cells was detected using MTT reduction assay.

[b] Relative viability was calculated by considering the survival of untreated cells (Control) as 100. Average absorbance value of the control was 0.61 at 540 nm.

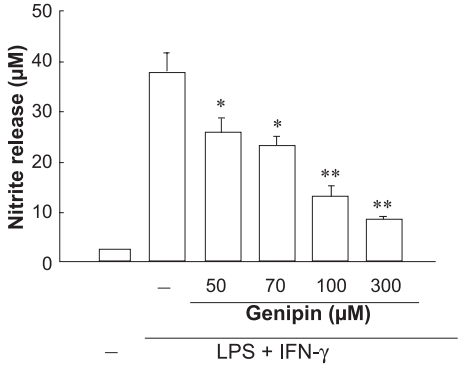


Fig. 4. Inhibitory effect of genipin on lipopolysaccharide (LPS) plus IFN-γ-induced NO production in RAW 264.7 cells. RAW 264.7 cells were incubated for 24 h with lipopolysaccharide (1 μg/ml) plus IFN-γ (10 U/ml) in the presence or absence of indicated concentrations of genipin. Accumulated nitrite in the culture medium was determined by the Griess reaction. The values are mean ± S.E. of four independent experiments. Asterisks indicate significant difference (**$P<0.01$; *$P<0.05$) compared with the only lipopolysaccharide plus IFN-γ-treated group by Duncan's multiple range test.

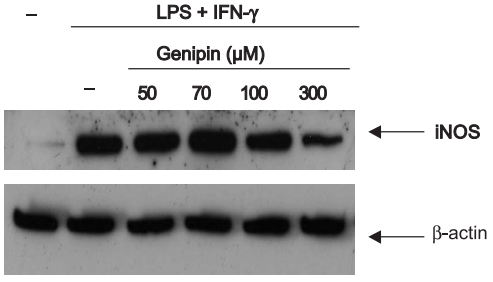


Fig. 5. Inhibitory effect of genipin on lipopolysaccharide (LPS) plus IFN-γ-induced expression of iNOS protein. RAW 264.7 cells were incubated with lipopolysaccharide (1 μg/ml) plus IFN-γ (10 U/ml) in the presence or absence of indicated concentrations of genipin. After 24-h incubation, cell lysates (30 μg protein) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and blotted with a mouse monoclonal anti-iNOS antibody. β-Actin was used as an internal control. This blot is a representative of three independent experiments.

### 3.5. Mediation of NF-κB in iNOS repression by genipin

As shown in Fig. 5, genipin significantly inhibited iNOS expression in RAW 264.7 cells stimulated with lipopolysaccharide plus IFN-γ. The activation of nuclear factor-κB (NF-κB) has been reported to be involved in NO production in lipopolysaccharide/IFN-γ-stimulated RAW 264.7 cells (Lowenstein et al., 1993). Because the nuclear translocation of NK-κB was found to depend on induced degradation of IκBs (Baldwin, 1996), the effect of genipin on lipopolysaccharide- or lipopolysaccharide/IFN-γ-induced IκB degradation was examined in RAW 264.7 cells. Lipopolysaccharide or lipopolysaccharide/IFN-γ caused reduced expression of IκB-β in untreated RAW 264.7 cells (Fig. 6). Genipin pretreatment markedly prevented the degradation of IκB-β in RAW 264.7 cells stimulated with both lipopolysaccharide and lipopolysaccharide/IFN-γ (Fig. 6). However, genipin was unable to prevent the degradation of IκB-α in stimulated RAW 264.7 cells (data not shown). These data suggest that genipin exerts the inhibition of iNOS expression via the specific prevention of IκB-β degradation.

Lipopolysaccharide has been reported to activate a series of mitogen-activated protein (MAP) kinases, such as extracellular signal-regulated kinases (Erk1/2), p38 and c-*Jun* $NH_2$-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs), in NO production (Ajizian et al., 1999). JNK/SAPK is known to activate c-*Jun*, a component of the transcription factor activation protein-1 (AP-1), by binding to the amino-terminal region of c-*Jun* and phosphorylating c-*Jun* at Ser63/73. To determine whether JNK/SAPK is involved in iNOS repression by genipin, its effect on JNK/SAPK activation was examined by measuring the phospho-c-*Jun* level (Ser63). Genipin did not reduce the phospho-c-*Jun* level, indicating that genipin was unable to inhibit JNK/SAPK activation (Fig. 7). Taken together, genipin is shown to inhibit iNOS expression via blocking the NF-κB pathway but not the JNK/SAPK pathway.

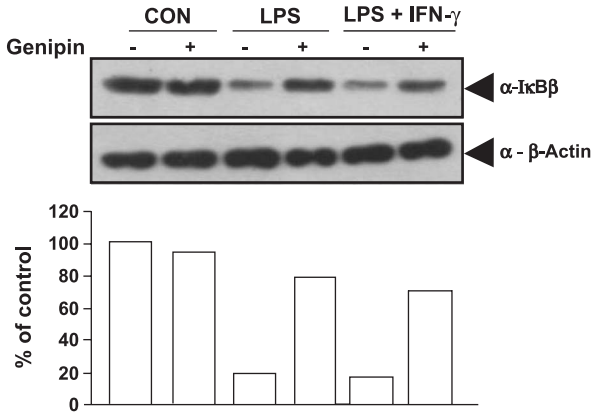


Fig. 6. Effect of genipin on IκB-β degradation in RAW 264.7 cells. The cells were pretreated with genipin (200 μM) for 30 min and incubated with lipopolysaccharide (LPS; 1 μg/ml) or lipopolysaccharide (LPS; 1 μg/ml)/IFN-γ (10 U/ml) for 15 min. CON indicates the RAW 264.7 cells not treated with lipopolysaccharide or lipopolysaccharide/IFN-γ. The cells were then lysed, and the lysates were subjected to SDS-PAGE and immunoblot analysis using anti-IκB-β antibodies. β-Actin was used as an internal control. A typical result of three independent experiments is shown. The lower panel shows relative band strength.

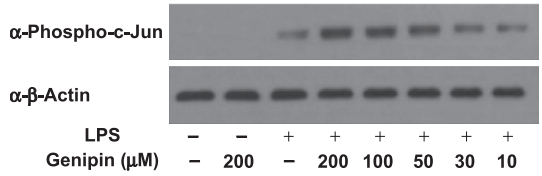


Fig. 7. Effect of genipin on JNK/SAPK activity in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. The cells were pretreated with genipin for 30 min and incubated with lipopolysaccharide (LPS; 1 μg/ml) for 15 min. The RAW 264.7 cells were then lysed, and the lysates were analyzed by immunoblotting using antibody to phopho-c-*Jun* (Ser63), a reaction product of the JNK/SAPK activity. β-Actin was used as an internal control. The data shown are representative of three independent experiments.

## 4. Discussion

In the present study, antioxidant activity of genipin was evaluated using three different assays. Genipin was found to possess a significant antilipoperoxidative capacity, but it did not show a free radical scavenging effect and an inhibition of xanthine oxidase activity. These results imply that genipin is able to effectively scavenge hydroxyl radicals formed in an $Fe^{2+}$/ascorbate system. However, geniposide did not show inhibitory activity in lipid peroxidation (data not shown). Crocetin and crocin, two other components of gardenia fruits, were previously shown to contain antioxidant activity (Hsu et al., 1999; Pharm et al., 2000). Crocetin is suggested to suppress the 12-*O*-tetradecanoylphorbol-13-acetate-induced skin carcinogenesis via its antioxidant property which in turn leads to a reduction in the 12-*O*-tetradecanoylphorbol-13-acetate-induced expressions of c-*Jun*, c-*Fos* and c-*Myc* in mouse epidermis (Hsu et al., 1999).

Antiangiogenic therapy may be an important component of treatment regimens for cancer patients. Several endogenous angiogenesis inhibitors including angiostatin (Gately et al., 1997) and endostatin (Sasaki et al., 1998) were identified. Angiogenesis inhibitors such as fumagillin and minocycline were identified from microbes (Ingber et al., 1990; Tamargo et al., 1991). Genipin was found to contain potent antiangiogenic activity using chorioallantoic membrane assay in this study. The antiangiogenic activity of genipin might support the antitumor effect of gardenia fruit, in addition to crocetin and penta-acetyl geniposide (Wang et al., 1992; Hsu et al., 1999). Penta-acetyl geniposide was obtained from the modified extract of gardenia fruits and is shown to be biologically active as an antitumor agent against C-6 glioma cells in culture (Wang et al., 1992). The antiangiogenic activity of genipin would be used as adjuvant or combination chemotherapy for the treatment of cancer.

The present results also demonstrate that genipin markedly decreased nitric oxide synthase (iNOS) expression in a low micromolar concentration range in RAW 264.7 macrophages. However, geniposide did not show any decreasing effects in the iNOS expression of RAW 264.7 magrophages (data not shown). Sugar component of geniposide may cause a difference in the effect on iNOS expression. Previously, geniposide was demonstrated to have less effect in preventing Alzheimer's amyloid β protein toxicity in cultured hippocampal neurons when compared to genipin (Yamazaki et al., 2001). Inhibition of iNOS expression by genipin may support its antiinflammatory and antiangiogenic activities. Genipin, one of the active principles in gardenia fruit, appears to be potent and a promising antiinflammatory and antiangiogenic agent.

Mammalian transcription factor NF-κB is a pleiotrophic regulator of various genes involved in inflammatory processes which controls the expression of proinflammatory cytokines and chemokines and modulates the molecular mechanisms involved in inflammation. NF-κB, a heterodimer of a 50- and a 65-kDa subunit, resides as an inactive cytosolic protein due to its interaction with inhibitory proteins of the IκB family in most cell types including blood monocytes (Mandrekar et al., 1999). NF-κB activation is induced by a variety of stimuli, such as mitogens, cytokines, lipopolysaccharide, viruses and UV light, which lead to the phosphorylation and the degradation of the IκB proteins (Baeuerle and Henkel, 1994; Mandrekar et al., 1999). IκB-α and IκB-β contain several ankyrin repeats responsible for binding NF-κB and two conserved serine residues (Fan et al., 2004). However, IκB-α with both nuclear import and export sequences has the ability to remove activated NF-κB from the nucleus, and IκB-β only binds to NF-κB in the cytoplasm (Fan et al., 2004). In the present study, we provide an evidence that genipin significantly prevents the degradation of IκB-β but not IκB-α in RAW 264.7 cells stimulated with both lipopolysaccharide and lipopolysaccharide/IFN-γ. This suggests that genipin shows antiinflammatory activity through the NF-κB/IκB-β pathway, which also correlates with the inhibitory activity of genipin on NO production and angiogenesis.

In conclusion, we have demonstrated that genipin possesses antilipoperoxidative and antiinflammatory activities. It is also shown to be an inhibitor of lipopolysaccharide plus IFN-γ-induced NO production via inhibiting iNOS expression and a potent antiangiogenic agent. These novel findings provide an additional pharmacological background on the efficacies of gardenia fruits.

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