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Identification of a new anti-LPS agent, geniposide, from *Gardenia jasminoides* Ellis, and its ability of direct binding and neutralization of lipopolysaccharide *in vitro* and *in vivo*

Xinchuan Zheng [a,1], Dong Yang [a,1], Xin Liu [a], Ning Wang [a], Bin Li [b], Hongwei Cao [a], Yongling Lu [a], Guo Wei [b], Hong Zhou [b,*], Jiang Zheng [a,*]

[a] Medical Research Center, Southwestern Hospital, Third Military Medical University, Chongqing 400038, China
[b] Department of Pharmacology, College of Pharmacy, Third Military Medical University, Chongqing 400038, China

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ABSTRACT

Lipopolysaccharide (LPS/endotoxin) is a key pathogen recognition molecule for sepsis. Currently, one of the therapeutic approaches for severe sepsis is focusing on the neutralization of LPS, and clinical trials have shown a lot of traditional Chinese herbs possess anti-sepsis function. Herein, to elucidate the bioactive components of traditional Chinese herbs that can neutralize LPS, the lipid A-binding abilities of sixty herbs were tested using affinity biosensor technology. The aqueous extract of *Gardenia jasminoides* Ellis, traditionally used to treat inflammation in Asian countries for centuries, was further investigated. Subsequently, a monomer, identified as geniposide, was isolated. *In vitro*, geniposide was found to directly bind LPS and neutralize LPS. It dose-dependently inhibited cytokines release from RAW264.7 cells induced by LPS without affecting the cell viability, and inhibited TNF-α mRNA expression up-regulated by LPS. However, geniposide did not decrease TNF-α release induced by CpG DNA, Poly I:C or IL-1β. Significantly, geniposide dose-dependently down-regulated TLR4 mRNA expression up-regulated by LPS, and suppressed the phosphorylations of p38 MAKP induced by LPS but not by IL-1β. *In vivo*, geniposide (40 mg/kg) could significantly protect mice challenge with lethal heat-killed *E. coli*, and dose-dependently decreased the level of serum endotoxin which was tightly associated with the cytokine levels in endotoxemia mice. In summary, we successfully isolated geniposide from *G. jasminoides* Ellis. Geniposide directly bound LPS and neutralized LPS *in vitro*, and significantly protected sepsis model mice. Therefore, geniposide could be as a useful lead compound for anti-sepsis drug development.

1. Introduction

Sepsis results in the activation of numerous proinflammatory mediators such as TNF-α, IL-1, IL-6 and IL-12, and this condition results in multiple organ dysfunction syndrome (MODS), septic shock and ultimately death [1,2]. The epidemiological study in the United States over the past decade found that the incidence of sepsis is at least 240 patients per 100,000 people [3]. Unfortunately, there are currently few effective adjuvant therapies in clinical use except activated protein C (APC) which targets the coagulation system [4]. However, APC is only recommended in patients at high risk of death (septic shock, sepsis-induced acute respiratory distress syndrome, acute physiology and chronic health evaluation II score of >/=25, and sepsis-induced multi-organ failure) without bleeding risk. Therefore, it is very urgent and important to investigate new anti-sepsis drugs.

Lipopolysaccharide (LPS/endotoxin), the major constituent of the outer membrane of gram-negative bacteria, is a common trigger of sepsis. The LPS-induced cytokine release leads to the pathophysiologic derangement associated with sepsis and septic shock. Lipid A, an evolutionarily conserved region of LPS, has been identified as the toxic component of LPS and therefore represents an ideal target for neutralization of LPS and anti-sepsis drug development [5,6].

During the past few years, there was a resurgence of interest in developing naturally occurring drugs from medicinal plants. Traditional Chinese herbs have been used for centuries as traditional remedies in China; their clinical uses are limited due to the unidentified complex substances of herbs, although a lot of traditional Chinese herbs possess anti-sepsis function [7]. They could reduce bacterial numbers during infection, decrease the level of serum LPS, inhibit LPS-stimulated cytokines release, and suppress infection-induced endotoxin shock in mice [8]. Significantly, some substances from these herbs had recently been shown to reduce TNF-α release by

* Corresponding authors. Zhou is to be contacted at Tel./fax: +86 23 6875 2266. Zheng, Tel.: +86 23 68754435; fax: +86 23 65460584.
E-mail addresses: zhouh64@163.com (H. Zhou), zhengj@mail.tmmu.com.cn (J. Zheng).
[1] They equally contribute to this work.

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directly binding to the lipid A moiety of LPS [8]. Therefore, we investigated the lipid A-binding abilities of herbs by using affinity biosensor technology, which had been established as a screening method for anti-sepsis agents' development in our lab in 2005 [9].

In this study, we demonstrated how an anti-sepsis monomer was screened and isolated from *Gardenia jasminoides* Ellis, and its anti-sepsis activity was investigated *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Geniposide (commercially standard substance) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (HPLC grade) was purchased from Honeywell Burdick & Jackson (Muskegon, USA). n-butyl alcohol and ethanol were purchased from Chuandong Chemical Factory (Chongqing, China). Macroporous adsorptive resins AB-8 was purchased from Haiguang Chemical Ltd. (Tianjin, China). Polyamide was purchased from Sijia Biochemical and Plastic Factory (Taizhou, China). All other chemicals were the best grade commercially available.

LPS from *E. coli* O111:B4, lipid A from *Salmonella Re* 595 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were purchased from Sigma Chemicals (St. Louis, MO, USA). Mouse TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Biosource International (Camarillo, CA, USA). The RNA easy kit (ReverTra Ace-α-) and Real-time PCR Master Mix (SYBR Green) were obtained from Toyoboco Ltd. (Osaka, Japan). The kinetic turbidimetric Limulus amebocyte lysate (LAL) kit was purchased from Zhanjiang A & C Biological Ltd. (Zhanjiang, China). Antibodies against p-p38, p38 and GAPDH were purchased from Santa Cruz Biotechnology (CA, USA). Recombinant murine IL-1β was obtained from PeproTech Inc. (Rocky Hill, NJ, USA). Poly I:C was purchased from Sigma Chemicals (St. Louis, MO, USA). CpG DNA was synthesized by SBS Genetech (Beijing, China).

2.1.2. Plant material

The dried fruit of *G. jasminoides* Ellis (gardenia fruit) was purchased in Chongqing, China, and identified in the Chongqing Academy of the Chinese Materia Medica (Chongqing, China).

2.1.3. Animals

Seventy-two KM mice (4–6 weeks old), with equal numbers of male and female, were obtained from the Experimental Animal Center of the Third Military Medical University (Chongqing, China). The weight of the mice on the day of the experiments was 20.5 ± 1.9 g. They were maintained in specific pathogen free (SPF) condition until used. All the experiments were conducted in accordance with the national guidelines for the care and use of laboratory animals.

2.1.4. Preparation of bacterial strain

E. coli ATCC35218 (EC) were kept in our laboratory and used for the mouse sepsis model. Single colonies from viable, growing LB agar plates were transferred to sterile liquid LB medium (containing 10 g of tryptone, 10 g of NaCl, and 5 g of yeast extract per liter) and cultivated in 50-ml volumes aerobically at 37 °C, shaking environmental chamber for 12 h. These cultures were then transferred to 500 ml of fresh LB medium for another 12 h. When bacteria were in the log phase of growth the suspension was centrifuged at 9391 ×*g* for 5 min at 4 °C, the supernatant was discarded, and the bacteria were resuspended and diluted into sterile saline to achieve a concentration of approximately 1×10^{10} colony formation units (CFU)/ml. Finally, bacterial suspension was incubated in a water bath at 100 °C for 30 min in order to inactivate the cells [10].

2.1.5. Cell line and culture

Murine macrophage RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% low endotoxin fetal calf serum (HyClone, Logan, UT), 2 μM glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml in a 37 °C humid atmosphere with 5% CO_2. The cells were diluted with 0.4% trypan blue in phosphate-buffered saline (PBS; 0.1 mM, pH 7.2), and live cells were counted with a hemacytometer.

2.2. Methods

2.2.1. Screening and purification

2.2.1.1. Screening of lipid A-binding herbs using affinity biosensor technology. Lipid A was immobilized on the surface of a hydrophobic cuvette according to the manufacturer's instructions (IAsys Affinity Sensors, Thermo Labsystem, USA), and as described previously [9].

Aqueous extracts from sixty herbs were screened for their abilities to bind lipid A. Among these herbs, *G. jasminoides* Ellis was selected for further investigated. Briefly, the herb was homogenized with distilled water. The aqueous extract (2 mg/ml) was filtered through a 0.22 μm membrane filter. Five microlitres of the extract was added into a cuvette containing 60 μl PBS/AE (a phosphate buffered saline containing 0.025% (w/v) sodium azide and 1 mM EDTA, pH 7.4). After 5 min, the cuvette was washed seven times with 60 μl PBS/AE and alternately washed with 0.1 M HCl, PBS/AE, and 10 mM NaOH, respectively. Data analyses were performed using the FASTplot software package (Thermo Labsystem, USA).

2.2.1.2. Extraction and isolation of lipid A-binding components from G. jasminoides Ellis using macroporous adsorptive resins and polyamide column chromatography. Gardenia fruit (3 kg) was homogenized with distilled water (9 L × 3). The extract was filtered and concentrated in a rotary evaporator (BUCHI Rotavapor R205, Switzerland), and then subjected to macroporous adsorptive resins AB-8 (10 cm i.d. × 150 cm) eluted with distilled water, 10% and 50% ethanol, respectively. The elute was collected and concentrated by rotary evaporation to yield three fractions which named Fractions A, B and C, respectively. Their binding activities to lipid A were assayed in turn as described as above. Fraction C possessed the highest binding activity to lipid A among them.

Fraction C dissolved in water was extracted by n-butyl alcohol. The extract was concentrated in a rotary evaporator to remove the n-butyl alcohol and then named as Fraction C2. Assaying for their binding activities to lipid A demonstrated that the n-butyl alcohol-soluble constituents showed a higher binding activity to lipid A than the water-soluble constituent named Fraction C1.

Fraction C2 was subjected to polyamide column chromatography (10 cm i.d. × 100 cm) eluted with distilled water, 20% and 50% ethanol. The elute was collected and concentrated by rotary evaporation, respectively, to yield three fractions which named as Fraction C2a, C2b and C2c, respectively. Five microlitres of each fraction (2 mg/ml) was assayed for its binding activity in turn. Fraction C2b was confirmed with the highest binding activity to lipid A.

2.2.1.3. Purification and identification of lipid A-binding monomer from G. jasminoides Ellis using high performance liquid chromatography (HPLC) technology. Fraction C2b was further purified by preparative RP-C18 HPLC using a ZORBAX SB-C18 PrepHT column (21.2 mm i.d. × 250 mm) with 7 μm particle size (Agilent Technologies, USA). The mobile phase was consisted of water and methanol (72:28, v/v). A compound was obtained and then named as CJ-1. The purity of CJ-1 was determined by HPLC and thin layer chromatography (TLC). CJ-1 was identified by analysis of physicochemical properties, TLC and spectrum, and also by comparison of those data with standard substance.

2.2.2. Bioactivity study in vitro

2.2.2.1. Affinity assay of CJ-1 for lipid A. As described above, lipid A was immobilized on the surface of a hydrophobic cuvette. Serial dilutions of CJ-1 (geniposide, 0.3, 0.6, 1.2, 2.4 and 4.8 mM) were added into the system, then a binding curve was generated, and the Kd value was measured with IAsys Affinity Sensors (Thermo Labsystem, USA). Data analyses were performed with the FASTplot and FASTfit software packages (Thermo Labsystem, USA).

2.2.2.2. Ability of CJ-1 to neutralize LPS. The LAL test, an extremely sensitive indicator of the free LPS, is usually performed at a low concentration of LPS. Using the LAL test, the ability of CJ-1 (geniposide) to neutralize LPS was assayed. Different concentrations of CJ-1 (geniposide, 0.25, 0.5, and 1.0 μg/ml) were incubated with LPS (0.1 ng/ml) at 37 °C for 30 min. Subsequently, 100 μl of this mixture was added to an equal volume of the LAL reagent. The kinetic turbidity was measured using EDS-99 Tube Reader (Zhanjiang A & C Biological Ltd., China).

2.2.2.3. Inhibition of TNF-α and IL-6 release induced by LPS and lipid A. The RAW264.7 cells (0.2 ml) were incubated in 96-well plate for 4 h, and the supernatants were then discarded and replaced with 0.2 ml serum-free DMEM. The cells were stimulated by LPS (100 ng/ml) or lipid A (2 μg/ml), CpG DNA (10 μg/ml), Poly I:C (20 μg/ml) or IL-1β (50 ng/ml); and CJ-1 (geniposide, 0, 50, 100, and 200 μg/ml) was added simultaneously. The cells were incubated for another 4 h and 12 h. The supernatants were collected to assess TNF-α and IL-6 levels using the appropriate ELISA kits.

2.2.2.4. Inhibition of TLR4 mRNA and TNF-α mRNA expression. The RAW264.7 cells (3.0 ml) were grown in 6-well polystyrene plates. The cells were stimulated by LPS (100 ng/ml); and CJ-1 (geniposide) was added at the indicated concentration (0, 50, 100 and 200 μg/ml). The cells were incubated for 4 h to extract the RNA. The method was previously reported in our lab [11], the inhibition of TLR4 and TNF-α mRNA expression were assayed using real-time quantitative PCR. The following primers were used: TLR4 (132-bp product), forward (5′-AGGCATGG-CATGGCTTACAC-3′) and reverse (5′-GGCCAATTTTGT CTCCACAGC-3′); TNF-α (217-bp product), forward (5′-CAGGTTCTGTCCCTTTCACTC ACT-3′) and reverse (5′-GTTCAGTAGACAGAAGAGCGTGGT-3′); mouse β-actin (191-bp product), forward (5′-GGGAAATCGTGCGTGACATCAAAG-3′) and reverse (5′-CATACCCAAGAAGGAAGGCTGGAA-3′).

2.2.2.5. Cytotoxicity assay. Cytotoxicity of CJ-1 (geniposide) was established using the MTT assay. RAW264.7 cells (0.2 ml) were grown in a 96-well plate, after an overnight culture period, cells were washed with DMEM and incubated with various concentrations of CJ-1 for 24 h. Subsequently, 20 μl of MTT solution (5 mg/ml in PBS) were added in a total volume of 200 μl medium. After cells were incubated for 4 h at 37 °C and 5% CO_2, the supernatant was removed and 150 μl DMSO was added to each well to dissolve produced formazan crystals. The extinction was measured at 490 nm using Model 550 microplate reader (Bio-Rad, USA).

2.2.2.6. Western blot analysis. The RAW264.7 cells (5×10^6/ml) grown in T75 flasks were stimulated by LPS (100 ng/ml) or IL-1β (50 ng/ml); and CJ-1 (geniposdie, 200 μg/ml) were added simultaneously. The

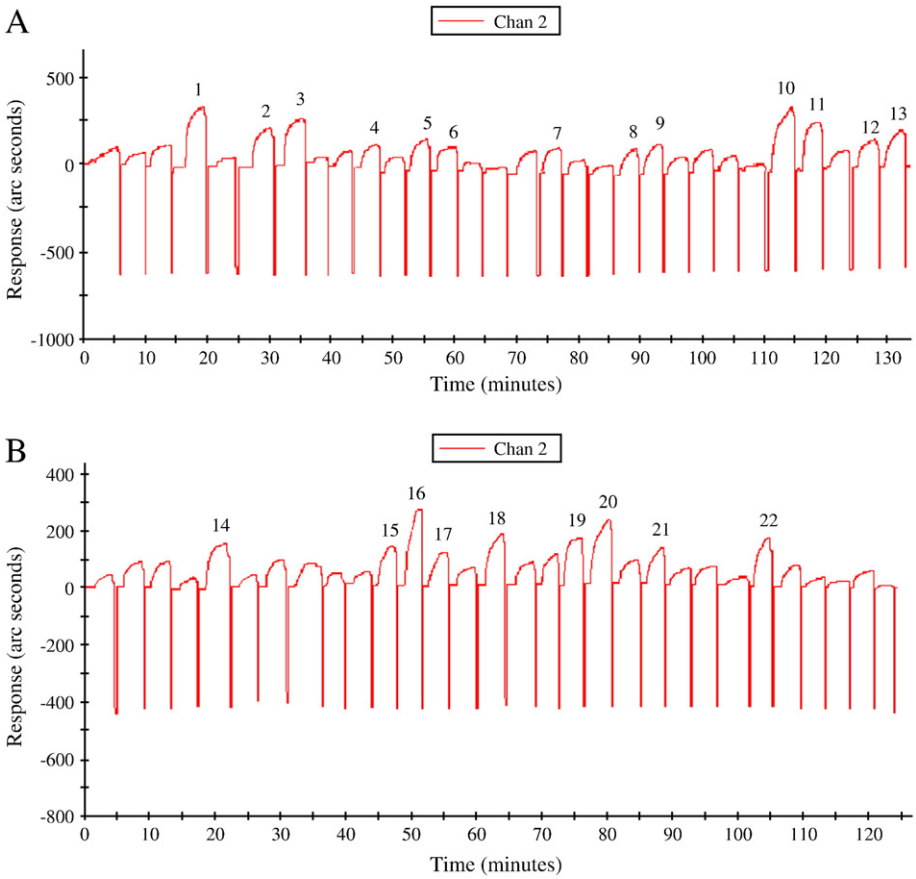

Fig. 1. Binding capacities of aqueous extracts of sixty Chinese herbs to lipid A. 1. *Picrorhiza scrophulariiflora* Pennell, 2. *Trichosanthes kirilowii* Maxim., 3. *Scutellaria baicalensis* Georgi, 4. *Cinnamomum cassia* Presl, 5. *Sanguisorba officinalis* L., 6. *Lycium chinense* Mill., 7. *Forsythia suspensa* (Thunb.) Vahl, 8. *Prunella vulgaris* L., 9. *Artemisia argyi* Levl. et Vant., 10. *Paeonia lactiflora* Pall., 11. *Terminalia chebula* Retz., 12. *Tripterygium wilfordii* Hook. f., 13. *Portulaca oleracea* L., 14. *Gardenia jasminoides* Ellis, 15. *Prunus mume* (Sieb.) Sieb. et Zucc., 16. *Melaphis chinensis* (Bell) Baker, 17. *Scrophularia ningpoensis* Hemsl., 18. *Canarium album* Raeusch., 19. *Stephania tetrandra* S. Moore, 20. *Poria cocos* (Schw.) Wolf, 21. *Amomum tsao-ko* Crevost et Lemarie, 22. *Paeonia suffruticosa* Andr.

Table 1
Binding activity of each fraction to lipid A.

	Response (arc seconds)							
	Fraction A	Fraction B	Fraction C	Fraction C1	Fraction C2	Fraction C2a	Fraction C2b	Fraction C2c
Lipid A	25	16	65	31	76	17	68	26

Immobilization of lipid A on the surface of a hydrophobic cuvette. Five microlitres of the extract was added into a cuvette containing 60 μl PBS/AE. After 5 min, the cuvette was washed seven times with 60 μl PBS/AE and alternately washed with 0.1 M HCl, PBS/AE, and 10 mM NaOH, respectively. Data analyses were performed using the FASTplot software package (Thermo Labsystem, USA).

cells were incubated for 1 h, and then lysed in 500 μl of lysis buffer containing RIPA, 1 mM PMSF and a cocktail of proteinase/phosphatase inhibitors (Biyuntian Biotech. Jiangsu, China) to obtain total protein which was separated by SDS-PAGE and electrophoretically transferred onto PVDF membranes (Millipore, MA, USA). The membranes were blocked in TBS buffer containing 0.05% Tween 20 and 5% non-fat dry milk for 1 h and incubated with the primary antibodies at 4 °C overnight, and then incubated with secondary antibodies for 1 h at 37 °C. Detection of binding antibodies was performed with Thermo Pierce SuperSignal West Femto Maximum Sensitivity Substrate Trial Kit (Rockford, IL, USA) and detected under a Bio-Rad ChemiDoc XRS (Hercules, CA, USA) gel imaging system.

2.2.3. Bioactivity study in vivo

2.2.3.1. Experimental sepsis animal models and treatment in mice. Thirty KM mice were randomly divided into three groups (10 mice/group) and were intravenously injected as follows: heat-killed *E. coli* (1.30×10^{11} CFU/kg) alone in group A, heat-killed *E. coli* (1.30×10^{11} CFU/kg) and immediate subsequent injection of CJ-1 (geniposide, 40 mg/kg) in group B, and CJ-1 (geniposide, 40 mg/kg) alone in group C. The total injection volume was 0.4 ml per 20 g bodyweight. The general conditions and mice mortalities were observed for 7 days.

2.2.3.2. Serum LPS level in endotoxemia mice. Fifteen KM mice were randomly divided into five groups (3 mice/group). Group 1 was injected with saline as a negative control; group 2 was given 50 μg/kg LPS; and groups 3, 4 and 5 were given 10, 20 and 40 mg/kg of CJ-1 (geniposide) followed by injection with LPS, respectively. The total injection volume was 0.2 ml per 20 g bodyweight. Mice were sacrificed at 30 min after inception of the experiment, and blood samples were collected from the heart. Twenty microlitres of the serum was diluted in 380 μl of saline. The LPS levels in the diluents were assayed by the LAL test.

2.2.3.3. Serum cytokine in endotoxemia mice assay. Twenty-seven KM mice were randomly divided into five groups. Group 1 (3 mice/group) was injected with saline as a negative control; group 2 (6 mice/group) was given 50 μg/kg LPS; and groups 3, 4 and 5 (6 mice/group) were given 10, 20 and 40 mg/kg of CJ-1 (geniposide) followed by injection with LPS, respectively. Mice were sacrificed at 2 h after inception of the experiment, and blood samples were collected for cytokines assessment.

2.2.4. Statistics and presentation of data

Each experiment was repeated at least twice. The Chi-squared exact test was used to analyze the differences in mouse mortality among the groups. Other results were expressed as mean ± standard deviation (S.D.). TNF-α, IL-6, and LPS data were analyzed with the one-way ANOVA test and post hoc Bonferroni correction was used for multiple comparisons. A *p*-value less than 0.05 was considered significant, and a *p*-value less than 0.01 was considered very significant.

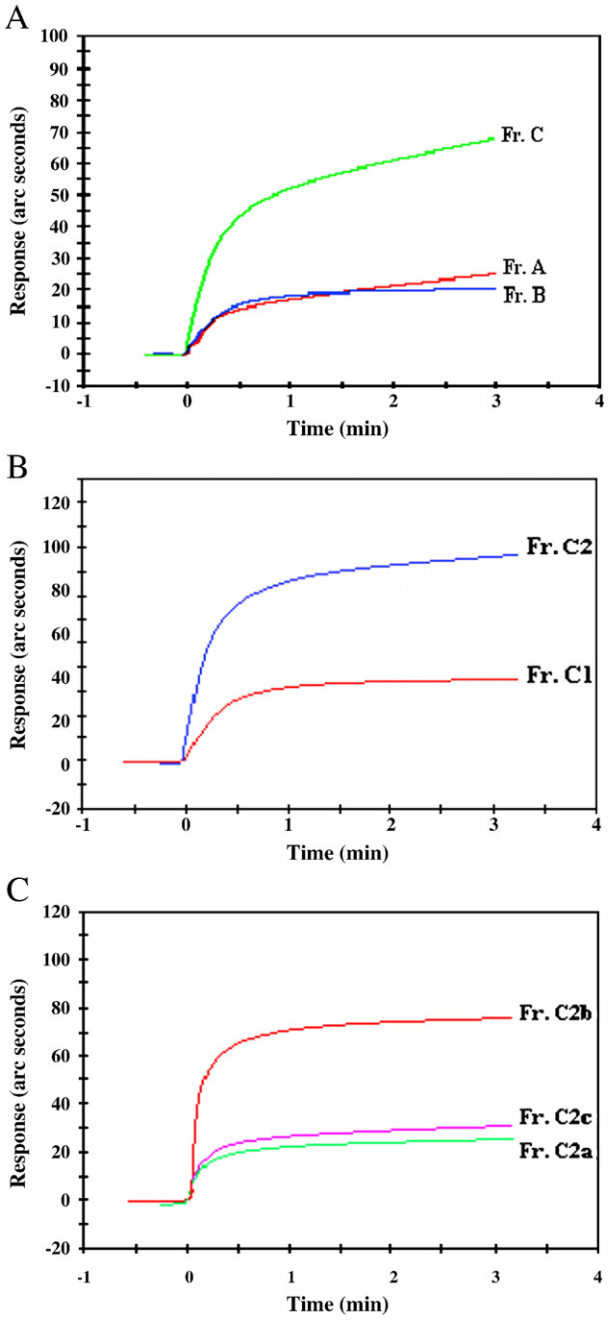

Fig. 2. Affinity assessment of fractions isolated from *Gardenia jasminoides* Ellis for lipid A. Each fraction was concentrated and filtered to make a concentration of 2 mg/ml. 5 μl of the fraction (Fr.) was added into a cuvette containing 60 μl PBS/AE. After 5 min, the cuvette was washed seven times with 60 μl PBS/AE and alternately washed with 0.1 M HCl, PBS/AE, and 10 mM NaOH, respectively. Data analyses were performed using the FASTplot software package.

3. Results

3.1. Screening and purification of geniposide

3.1.1. G. jasminoides Ellis is selected from sixty traditional Chinese herbs

Among sixty tested traditional Chinese herbs (data not shown), twenty-two herbs were found to possess higher lipid A-binding activities (RU>100″) (Fig. 1A and B). *G. jasminoides* Ellis was selected for its higher affinity for lipid A and low content of tannin (the tannin-gelatin precipitation assay was negative) that was unsuitable for intravenous injection. Therefore, *G. jasminoides* Ellis was investigated in the subsequent experiments.

3.1.2. A lipid A-binding fraction is isolated from G. jasminoides Ellis

Three fractions named Fractions A, B, C were isolated from *G. jasminoides* Ellis by macroporous adsorptive resins AB-8 and polyamide column chromatography. And then the abilities of Fractions A, B, C were tested using LAL test, but LAL test could be interfered by the complicated chemical components such as polysaccharides in the herb (data not shown). So LAL test was unsuitable for testing the ability of complicated

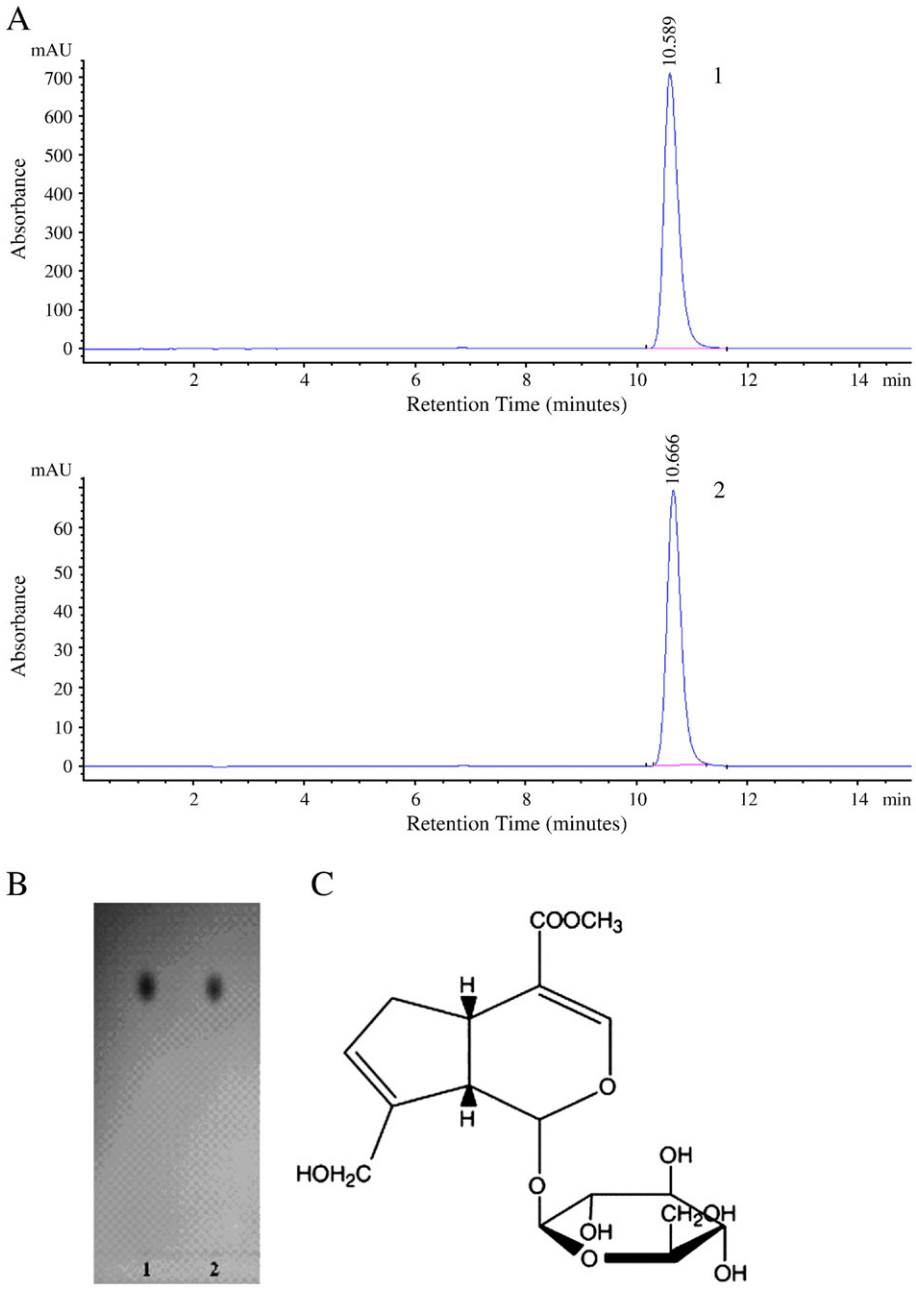

Fig. 3. Isolation and identification of CJ-1 as geniposide. (A) HPLC chromatogram with diode array detection (190–400 nm). Purified geniposide (retention time = 10.589 min) was analysed by HPLC using an Eclipse XDB C-18 column (4.6 mm i.d. × 150 mm) with 5 μm particle size (Agilent Technologies, USA). The temperature of the column was maintained at 35 °C. The following gradient system was used (min/% methanol): 0/28, 12/28, 19/100 for detection. UV spectra were recorded in the 190–400 nm range and the chromatograms were acquired at 240 nm. The mobile phase was pumped through the column at 1 ml min^{-1} before which the column was washed with 28% methanol for 60 min. The data was collected and analyzed by Agilent ChemStation software. 1. CJ-1 (retention time = 10.589 min); 2. geniposide (retention time = 10.666 min, commercially standard substance). (B) TLC chromatogram. The experiment was carried out on silica gel G plates with acetone/ethyl acetate/acetic acid/water (5:5:1:1, v/v/v/v) as the developing solvent, and methanol/sulphuric acid (1:1, v/v) as developer. Geniposide had Rf value of 0.67 in TLC. 1. CJ-1; 2. Geniposide (commercially standard substance). (C) Chemical structure of geniposid.

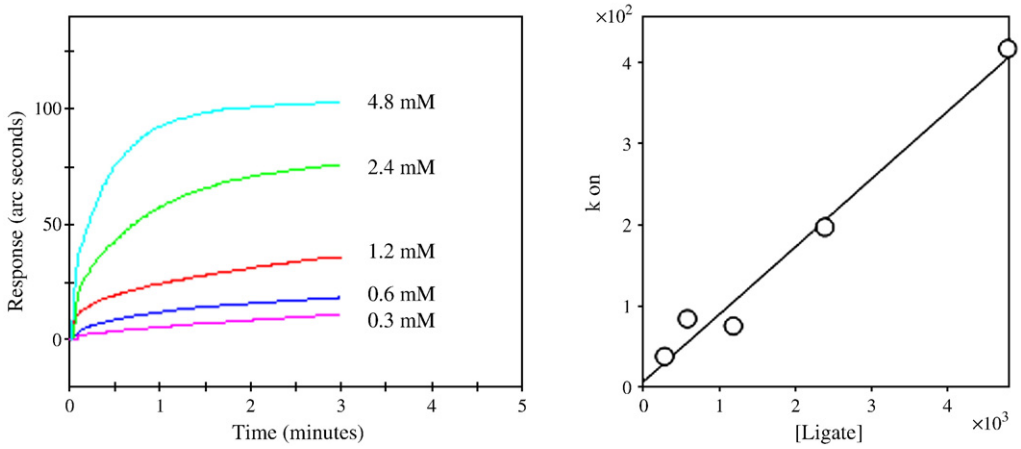

Fig. 4. Affinity of geniposide for lipid A. Lipid A was immobilized on the surface of a hydrophobic cuvette as described previously. Different concentrations of geniposide (in a range from 0.3 to 4.8 mM) were added to the immobilized cuvette, then a binding curve was generated and Kd value was measured and calculated.

crude extract to neutralize LPS. In contrast, affinity biosensor technology was not interfered by the complicated chemical components and to be used as a more convenient screening method.

Among Fractions A, B, and C, the binding ability of Fraction C to lipid A was higher than the others (Table 1, Fig. 2A). Thus, Fraction C was extracted by n-butyl alcohol. The n-butyl alcohol-soluble constituent (Fraction C2) showed higher binding activity to lipid A than the water-soluble constituents (Fraction C1) (Table 1, Fig. 2B).

Fraction C2 was further subjected to polyamide column chromatography. Subsequently, a fraction (Fraction C2b) with the highest binding ability to lipid A (Table 1, Fig. 2C) was collected and further purified by preparative RP-C18 HPLC.

3.1.3. A lipid A-binding monomer, geniposide, is isolated from Fraction C2b

Fraction C2b was subjected to preparative HPLC eluted with 28% methanol to yield a compound (CJ-1). The purity of CJ-1 was more than 98% determined by HPLC (Fig. 3A) and TLC (Fig. 3B). Then CJ-1 was identified as geniposide (Fig. 3C) by analysis of physicochemical properties, TLC and spectrum as reported previously [12], and also by comparison of those data with standard substance.

Geniposide ($C_{17}H_{24}O_{10}$) was a fine white powder. Molish reaction was positive. Melting point (m.p.) was 163–165 °C. UV λ_{max} (CH_3OH) nm was 240 nm. IR (KBr) cm^{-1} were 3450, 1710, 1640.

3.2. Bioactivity study of geniposide in vitro

3.2.1. Geniposide has a higher affinity for lipid A

FASTplot was used to generate binding curves, and the Kd value was measured and calculated by FASTfit (Thermo Labsystem, USA). Geniposide bound lipid A with Kd value of 6.77×10^{-5} M (Fig. 4).

3.2.2. Geniposide neutralizes LPS in vitro

Geniposide itself had no direct influence on the reaction of the LAL. However, 0.25, 0.5 and 1.0 μg/ml of geniposide neutralized 18.9%, 31.2% and 56.1% of LPS (0.1 ng/ml), respectively (Fig. 5). Above results showed, geniposide neutralized LPS in a dose-dependent manner *in vitro*.

3.2.3. Geniposide inhibits TNF-α mRNA expression, and TNF-α and IL-6 release from RAW264.7 cells stimulated by LPS and lipid A

Cytokines such as TNF-α and IL-6 play key roles in sepsis [13]. To confirm whether geniposide's neutralizing-LPS effect was paralleled with an inhibitory effect on cytokines release induced by LPS, the ability of geniposide to inhibit TNF-α and IL-6 release from RAW264.7 cells stimulated by LPS and lipid A was investigated. Herein, the influence of geniposide on TNF-α release induced by CpG DNA, Poly I:C or IL-1β were also observed in order to confirm whether geniposide's effect is related to its specifically binding and neutralizing LPS. The results demonstrated that LPS and lipid A could significantly induce RAW264.7 cells to release TNF-α and IL-6, but geniposide (50, 100 and 200 μg/ml) significantly inhibited cytokines release induced by both LPS and lipid A in a dose-dependent manner. However, geniposide (100 and 200 μg/ml) couldn't decrease TNF-α release induced by CpG DNA, Poly I:C or IL-1β (Fig. 6). Above results demonstrated that geniposide only decrease cytokines release induced by LPS not by other stimuli. In addition, we also found geniposide down-regulated the TNF-α mRNA expression stimulated by LPS in RAW264.7 cells using real-time quantitative PCR method (Fig. 7).

3.2.4. Geniposide reduces TLR4 mRNA expression up-regulated by LPS

TLR4 signaling is required for the induction of cytokine release by LPS [14,15]. To investigated whether geniposide could affect TLR4 signaling, the expression of TLR4 mRNA was observed using real-time quantitative PCR method. The results showed TLR4 mRNA was expressed at a very low level in non-stimulated cells, but LPS markedly up-regulated TLR4 mRNA expression. Significantly,

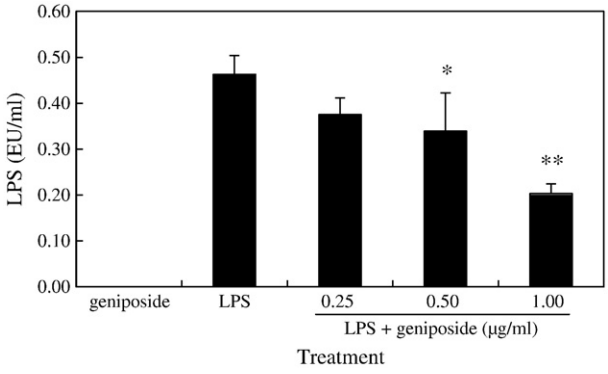

Fig. 5. LPS-neutralization of geniposide in a dose-dependent manner *in vitro*. Indicated concentrations of geniposide were incubated with LPS (0.1 ng/ml) at 37 °C for 30 min. Subsequently, 100 μl of this mixture was added to an equal volume of the LAL reagent and the kinetic turbidity was measured. The test was repeated three times. $^{*}p<0.05$ and $^{**}p<0.01$ as compared to the treatment without geniposide.

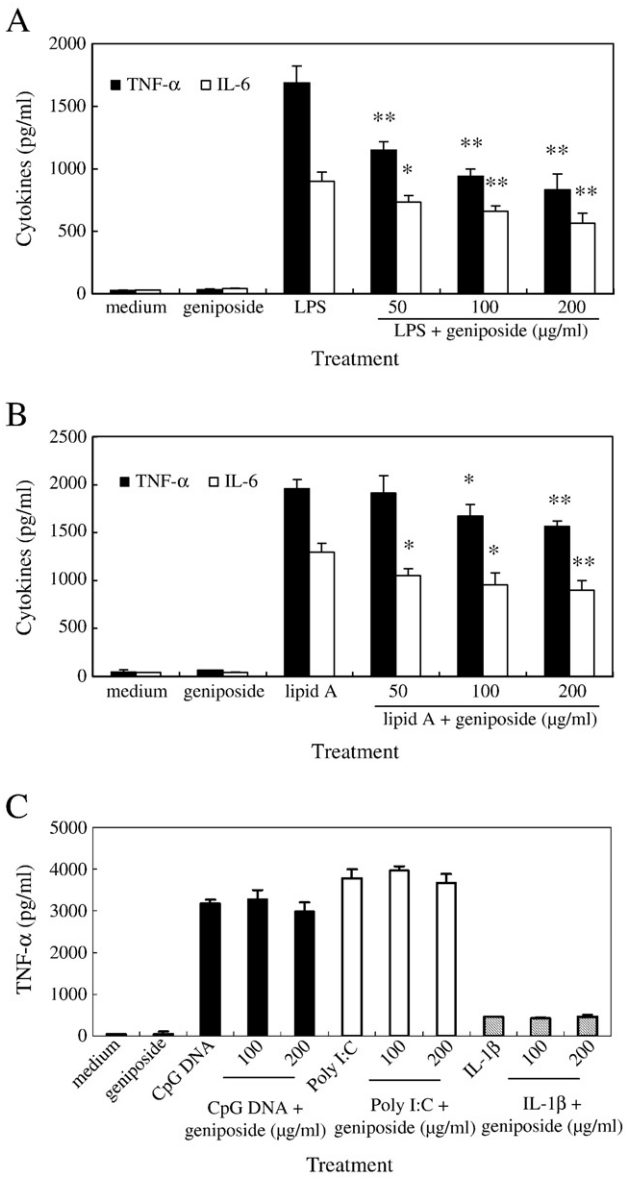

Fig. 6. Inhibition of geniposide on TNF-α and IL-6 release from RAW264.7 cells induced by LPS (A) or lipid A (B) in a dose-dependent manner. RAW264.7 (0.2 ml) were incubated in 96-well plate for 4 h, and the supernatants were then discarded and replaced with 0.2 ml serum-free DMEM. Then the cells were stimulated with LPS (100 ng/ml), lipid A (2 μg/ml), CpG DNA (10 μg/ml), Poly I:C (20 μg/ml) or IL-1β (50 ng/ml), and indicated concentrations of geniposide was added simultaneously. After cells were incubated for another 4 h and 12 h, the levels of TNF-α and IL-6 in the supernatants were analyzed using the appropriate ELISA kits. $^{*}p<0.05$ and $^{**}p<0.01$ as compared to only LPS or lipid A treatment.

geniposide (50, 100 and 200 μg/ml) could down-regulate the TLR4 mRNA augmentation (Fig. 8) ($p<0.01$).

3.2.5. Geniposide has no cytotoxicity in vitro

To exclude the possibility of geniposide affecting the cell viability, the cytotoxicity on RAW264.7 cells were investigated using the MTT assay. Geniposide (50, 100, 200 and 400 μg/ml) treatment for 24 h did not produce cytotoxicity on RAW264.7 cells (data not shown). The concentration of geniposide used in above experiments was less than 400 μg/ml, and incubation time was less than 24 h, therefore there was no correlation between the geniposide's inhibition on cytokine release and its cytotoxicity.

3.2.6. Geniposide suppresses p38 MAPK phosphorylation induced by LPS

p38 MAPK is a very important kinase for all of TLRs- and IL-1β-mediated pathway. Therefore, the effect of geniposide on phosphorylation level of p38 in RAW264.7 cells induced by LPS or IL-1β was measured by western blot. The results showed LPS (100 ng/ml) or IL-1β (50 ng/ml) alone markedly activated phosphorylation of p38, but geniposide treatment (200 μg/ml) could suppress the phosphorylated level of p38 induced by LPS but not by IL-1β (Fig. 9).

3.3. Bioactivity study of geniposide in vivo

3.3.1. Geniposide protects mice against a lethal dose heat-killed E. coli challenge

To test whether geniposide could protect mice from a lethal challenge with heat-killed *E. coli*, mice were injected with different doses of geniposide prior to a lethal challenge with heat-killed *E. coli* (1.30×10^{11} CFU/kg, i.v.).

For mice challenged by heat-killed *E. coli*, 90% of the mice died within 24 h. By contrast, geniposide-pretreatment (40 mg/kg) protected mice from heat-killed *E. coli* challenge ($p<0.01$). The result demonstrated that geniposide had a significant protection on sepsis animals (Fig. 10).

3.3.2. Geniposide decreases serum LPS level and inhibited cytokine release in endotoxemia mice

To test whether geniposide could neutralize LPS activity *in vivo*, the serum LPS concentration in the endotoxemia mice treated with or without geniposide were tested. In the endotoxemia mice, the serum LPS level sharply increased, but geniposide ranging from 10 to 40 mg/kg significantly reduced the serum LPS level in a dose-dependent manner (Fig. 11).

To determine whether the protective effect of geniposide was associated with cytokine reduction *in vivo*, we tested the serum cytokine levels in endotoxemia mice. Our previous experiments showed TNF-α and IL-6 levels peaked at 2 h after mice were injected with sublethal doses of LPS and geniposide did not delay the cytokine peak (data not shown). Therefore, mice were sacrificed at 2 h in the present experiments. Our results showed TNF-α and IL-6 levels significantly increased in mice treated with LPS, but geniposide-treatment (40 mg/kg) reduced peak TNF-α and IL-6 levels (Fig. 12), which was associated with the reduction of endotoxin level.

4. Discussion

In the present study, we reported that geniposide, isolated from *G. jasminoides* Ellis under the direction of bioactivities, is a novel natural anti-LPS agent. *In vitro*, geniposide could neutralize LPS and inhibit proinflammatory cytokines release from RAW264.7 cells induced by both LPS and lipid A. *In vivo*, geniposide protected mice against a lethal challenge with heat-killed *E. coli* and significantly decreased the levels of serum LPS paralleled that of cytokines in endotoxemia mice. To the best of our knowledge, this is the first report to demonstrate the anti-sepsis activity of geniposide.

As a plant belonged to *Rubiaceae* family, gardenia is well known to contain various iridoid glycosides which are unstable, non-volatile and thermally labile polar natural compounds [16,17]. The fruit of *G. jasminoides* Ellis (gardenia fruit) is an oriental folk medicine. It was widely used for treatment of inflammation, jaundice, headache, edema, fever, hepatic disorders and hypertension for centuries [18–21]. Iridoid compounds, geniposide and genipin, were found to be the anti-inflammatory components of the fruit. Both geniposide and genipin played anti-inflammatory activities via their inhibiting production of exudate and nitric oxide (NO) in carrageenan-induced rat paw edema model. Genipin suppressed both NO production and cyclooxygenase expression at an early stage, and possessed stronger anti-inflammatory activity than geniposide [22]. The genipin's inhibition on NO production

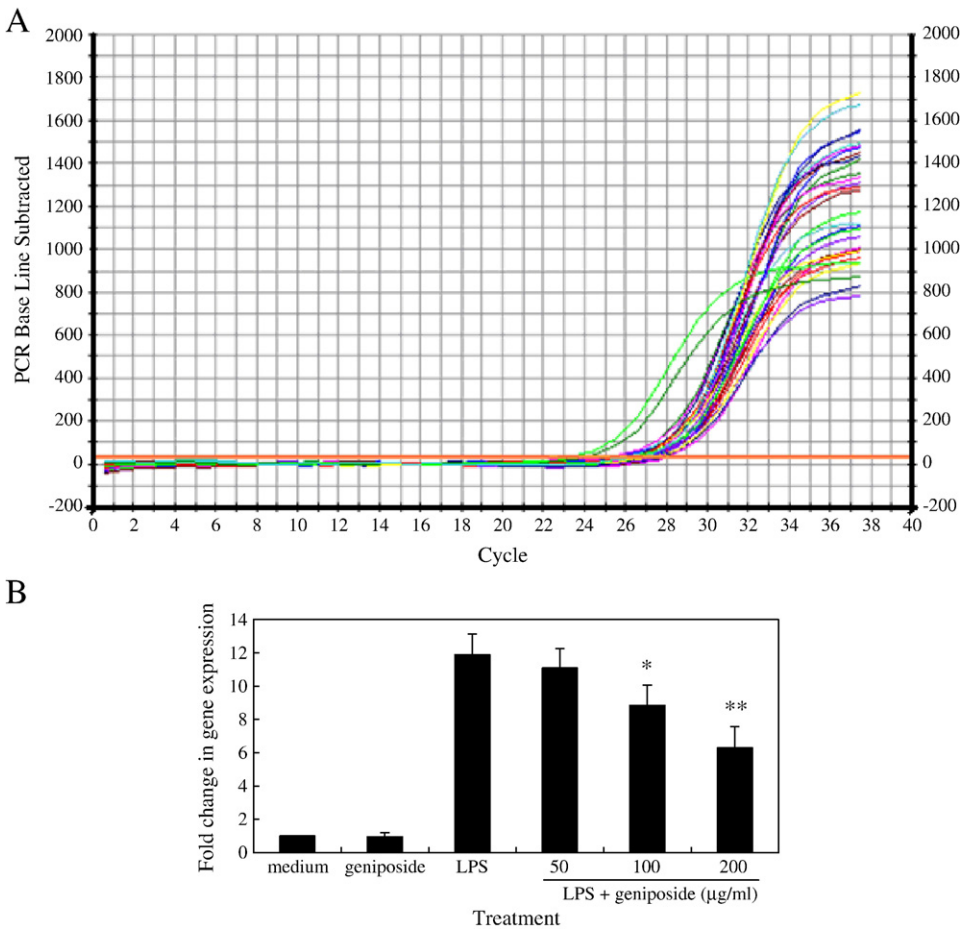

Fig. 7. Effect of geniposide on TNF-α mRNA expression in RAW264.7 cells stimulated by LPS. RAW264.7 cells incubated with 0.2 ml serum-free DMEM were stimulated with LPS (100 ng/ml), and geniposide was added at the indicated concentration (0, 50, 100 and 200 μg/ml). The cells were incubated for 4 h and then extract the mRNA. The amplification plot of real-time quantitative PCR were shown in (A). The fold changes of TNF-α mRNA expression were analyzed by the $2^{-\Delta\Delta CT}$ method (B). For the untreated sample (medium), ΔΔCT equals zero and 2^0 equals 1. So that the fold changes in gene expression relative to the untreated sample equals 1, by definition. For the treated samples (treated by LPS and serial dilutions of geniposide), evaluation of $2^{-\Delta\Delta CT}$ indicated the fold change in gene expression relative to the untreated sample. * $p<0.01$ as compared to only LPS treatment.

was associated with the inhibition of NF-κB activation [23]. However, nowadays, the anti-inflammatory material basis of gardenia fruit is not completely clear.

Previously, we immobilized lipid A on the hydrophobic cuvette of biosensor as the target to screen lipid A-binding agents, and obtained 1,2,3,4,6-β-D-pentagalloylglucose (PGG) and 2′,5,6′,7-tetrahydroxy-flavanonol (THF) from medicinal plants [9,11]. Both PGG and THF belong to phenols compounds according to their chemical structure. In our present experiments, gardenia fruit showed its potential as a lipid A-binding agent, so we investigated the aqueous chemical constituents which possessed the highest binding ability to lipid A among the three extracts (water, ethanol and petroleum ether). We isolated geniposide under the direction of bioactivity using affinity biosensor technology although geniposide probably was not the only compound binding to lipid A.

Geniposide had been purified from *G. jasminoides* Ellis in 1969 and Vietnamese *Paederia scandens* in 2002 [24,25], but this was the first time to discover that geniposide could bind LPS with Kd value of 6.77×10^{-5} M and possessed anti-sepsis activities in the present experiments. Significantly, the structure characteristic of geniposide is quite different from those of PGG and THF. There is no positive charge in the molecule of geniposide that is not like the compounds such as polymyxin and bactericidal/permeability increasing protein (BPI). Polymyxin and BPI could bind to LPS with large negative charge and then lead to the loss of LPS activity [26]. Therefore, how geniposide to interact with LPS is unclear. The binding site of geniposide on lipid A and conformational change following binding might be worth investigating in the future.

Excessive release of proinflammatory cytokines such as TNF-α, IL-6 and IL-12 induced by LPS lead to the pathophysiologic derangement associated with sepsis and septic shock [27], so it is very important to inhibit proinflammatory cytokines release and restore the balance of proinflammatory cytokines and anti-inflammatory cytokines. Herein, our data demonstrated that geniposide not only bound LPS but also decreased TNF-α and IL-6 release from RAW264.7 cells induced by LPS or lipid A in a dose-dependent manner. The inhibitory effects of geniposide on LPS-induced cytokine release were unlikely due to its nonspecific cellular toxicity because we did not observe cytotoxicity of geniposide on RAW264.7 cells at the concentrations used in the experiment.

TLRs-mediated signaling represents a principal molecular pathway for host innate immunity [28]. Mechanistically, it can be segregated into two distinct cascades: the MyD88-dependent and MyD88-independent cascades. MyD88-dependent signaling is common for all the TLRs, except TLR3, which exclusively utilizes the MyD88-independent pathway. Crosstalk between different TLRs-mediated pathways and the usage of multiple adaptors, kinases and transcription factors generates diversity to the pathway. There is crosstalk between TLRs-mediated pathways. p38 MAPK is a very important crosstalk kinase for all of TLRs-mediated pathways [29]. TLR4 is a pattern recognition receptor for LPS. LPS activates the TLR4-mediated signal transduction pathway to regulate the release of cytokines [14,15]. Herein, our results

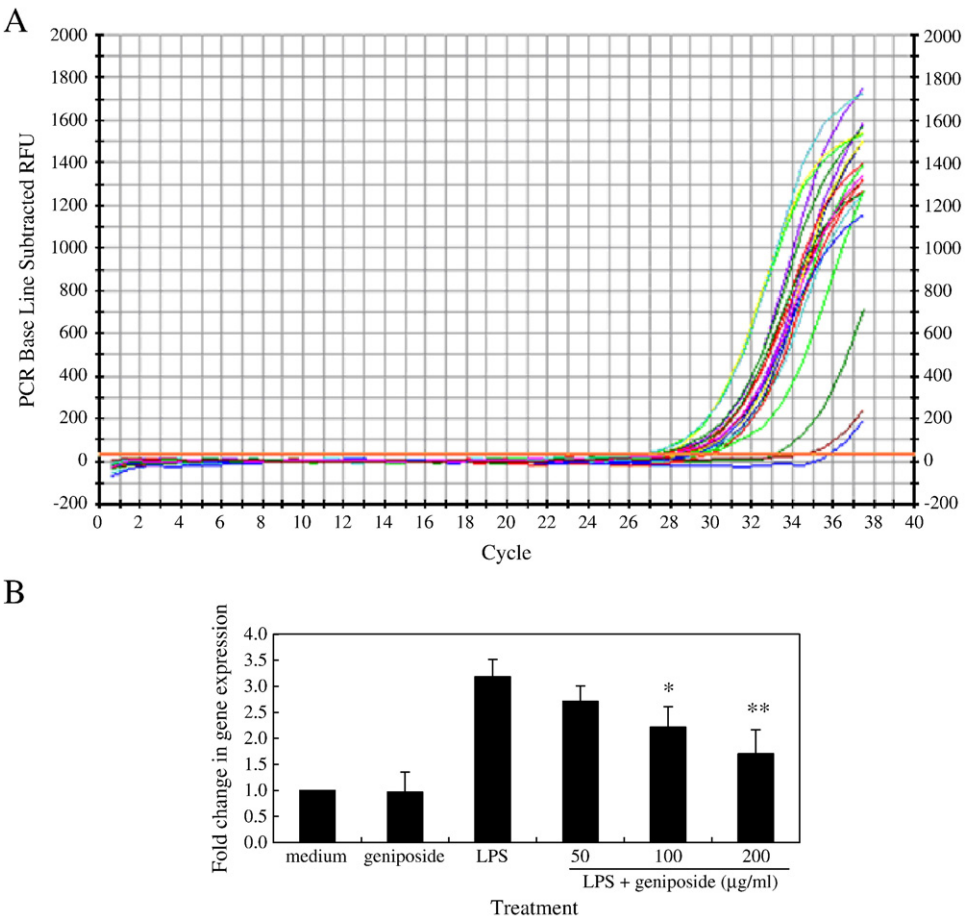

Fig. 8. Effect of geniposide on TLR4 mRNA expression in RAW264.7 cells stimulated by LPS. RAW264.7 cells incubated with 0.2 ml serum-free DMEM were stimulated with LPS (100 ng/ml), and geniposide was geniposide was added at the indicated concentration (0, 50, 100 and 200 μg/ml). The cells were incubated for 4 h and then extract the mRNA. The amplification plot of real-time quantitative PCR were shown in (A). The fold changes of TLR4 mRNA expression were analyzed by the $2^{-\Delta\Delta CT}$ method (B). For the untreated sample (medium), ΔΔCT equals zero and 2^0 equals 1. So that the fold changes in gene expression relative to the untreated sample equals 1, by definition. For the treated samples (treated by LPS and serial dilutions of geniposide), evaluation of $2^{-\Delta\Delta CT}$ indicated the fold change in gene expression relative to the untreated sample. * $p<0.01$ as compared to only LPS treatment.

showed geniposide could dose-dependently down-regulate the TLR4 mRNA expression up-regulated by LPS and phosphorylation of p38 MAPK induced by LPS. Above results suggested geniposide's down-regulation of TLR4 mRNA expression and inhibition of phosphorylation of p38 MAPK probably was the subsequent event after LPS was bound and neutralized by geniposide.

Importantly, in our *in vivo* mouse experiments, geniposide (40 mg/kg) protected mice against a lethal dose heat-killed *E. coli* challenge. At 30 min after LPS injection, geniposide (10, 20 and 40 mg/kg) dose-dependently reduced the peak LPS level in endotoxemia mice. However, only higher doses of geniposide (40 mg/kg) reduced the peak TNF-α and IL-6 levels in endotoxemia mice.

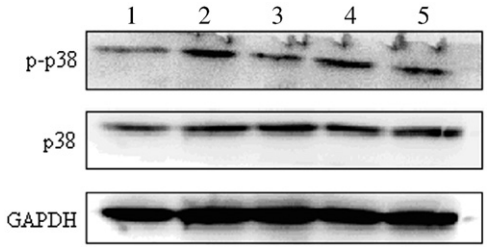

Fig. 9. Effect of geniposide on LPS- and IL-1β-induced p38 phosphorylation in RAW364.7 cells. The RAW264.7 cells were stimulated by LPS (100 ng/ml) or IL-1β (50 ng/ml), and geniposdie (200 μg/ml) were added simultaneously. The cells were incubated for 1 h, and then lysed to obtain total protein which was determined by western blot. Lane 1, medium; Lane 2, LPS; Lane 3, LPS + geniposdie; Lane 4, IL-1β; Lane 5, IL-1β + geniposdie.

The affinity of geniposide for LPS (6.77×10^{-5} M) was not very high, so there was another mechanism involving in its inhibitory effect on cytokines release induced by LPS? The answer was not.

Firstly, as well-known, affinity sensor technology is a well-established optical biosensor technology based on surface plasmon

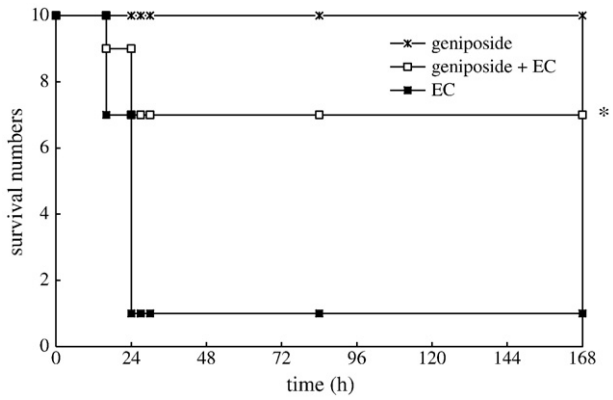

Fig. 10. Protection of geniposide on mice challenged with a lethal dose heat-killed *E coli*. Thirty mice were randomly divided into three groups ($n=10$). Groups of mice were treated with heat-killed *E. coli* (EC, 1.30×10^{11} CFU/kg) alone, geniposide (40 mg/kg) and EC, geniposide (40 mg/kg) alone. The mice were observed for 168 h. *$p<0.01$ as compared to EC group.

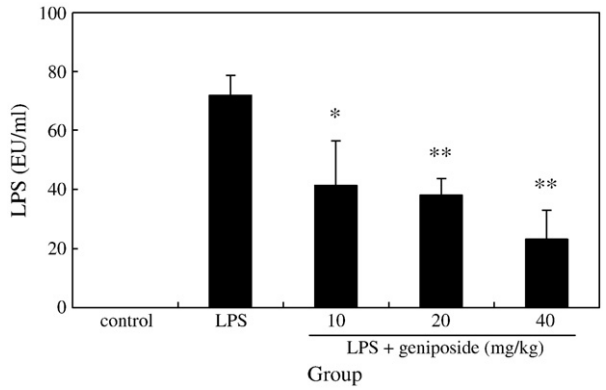

Fig. 11. Reduction of geniposide on serum LPS levels in endotoxemia mice. Fifteen mice were randomly divided into five groups (3 mice/group). Group 1 was injected with saline as a negative control; group 2 was given 50 μg/kg of LPS; and groups 3, 4 and 5 were given 10, 20 and 40 mg/kg of geniposide followed by injection with LPS, respectively. The total injection volume was 0.2 ml/20 g bodyweight. Mice were sacrificed at 30 min after inception of the experiment and blood samples were collected from the heart. Twenty microlitres of the serum was diluted in 380 μl of saline. The endotoxin level in the diluents was assayed by the LAL test. The data points are presented as mean ± standard deviation. $^{*}p<0.05$ and $^{**}p<0.01$ as compared to LPS group.

resonance (SPR) for detection of interaction between molecules. This technology could distinguish specific binding from non-specific binding [30,31]. Secondly, in the present experiments, geniposide only inhibited cytokines release induced by LPS and had no inhibitory effect on TNF-α release induced by CpG DNA, Poly I:C or IL-1β, thus demonstrating the effect of geniposide was specific for LPS. Thirdly, p38 MAPK pathway is common for proinflammatory cytokines release. CpG DNA, Poly I:C or IL-1β as well as LPS could activate p38 MAPK phosphorylation. Previously, geniposide was reported to block the activation of NF-κB, degradation of IκB-α, and phosphorylation of p38 MAPK and ERK1/2 in HUVECs challenged by LPS [32]. Herein, geniposide was also discovered to suppress the p38 phosphorylation induced by LPS, but didn't suppress p38 phosphorylation induced by IL-1β, further demonstrating the effect of geniposide was specific for LPS. Combination with above data, we confirmed that the effect produced by geniposide was mainly due to its binding and neutralization of LPS.

In conclusion, geniposide was isolated from gardenia fruit under the direction of bioactivity using affinity biosensor technology. Geniposide could significantly protect mice against a lethal dose heat-killed *E. coli* challenge, and this protection was tightly associated with its binding and neutralizing LPS and then inhibition on cytokines release.

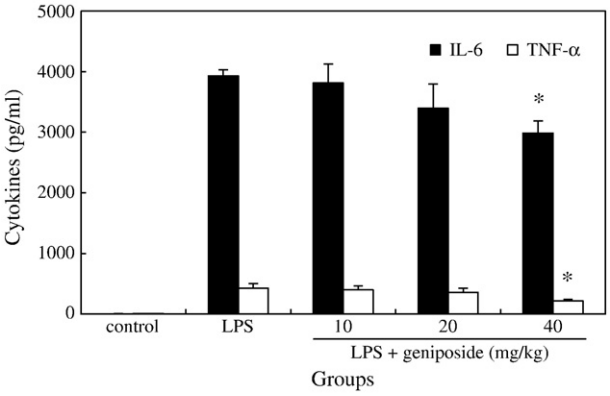

Fig. 12. Inhibition of geniposide on serum cytokines release in endotoxemia mice. Twenty-seven mice were randomly divided into five groups. Group 1 (3 mice/group) was injected with saline as a negative control; group 2 (6 mice/group) was given 50 μg/kg LPS; and groups 3, 4 and 5 (6 mice/group) were given 10, 20 and 40 mg/kg of geniposide followed by injection with LPS, respectively. Mice were sacrificed at 2 h after inception of the experiment, and blood samples were collected for cytokines assessment. $^{*}p<0.01$ as compared to LPS group.

Acknowledgements

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