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# An anti-sepsis monomer, 2′,5,6′,7-tetrahydroxyflavanonol (THF), identified from *Scutellaria baicalensis* Georgi neutralizes lipopolysaccharide *in vitro* and *in vivo*

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ABSTRACT

Lipopolysaccharide (LPS) is a known trigger in the pathogenesis of sepsis, lipid A being the toxic component. One of several adjuvant therapeutic approaches for severe sepsis is currently focusing on the neutralization of LPS. In order to obtain the components from traditional Chinese herbs that can neutralize the endotoxin, aqueous extractions of twelve herbs were tested using affinity biosensor technology. From twelve herbs, *Scutellaria baicalensis* Georgi (Huang Qin) found to possess high lipid A-binding abilities, and was selected in subsequent experiments. After subjected to macroporous adsorptive resins and HPLC, we obtained 2′,5,6′,7-tetrahydroxyflavanonol (THF) from *S. baicalensis* Georgi under the direction of neutralization of LPS and reducing proinflammatory cytokines. *In vitro*, THF directly bound to LPS and neutralized its activity. THF not only down-regulated TNF-α mRNA expression but also decreased TNF-α and IL-6 release from RAW264.7 cells induced by LPS in a dose-dependent manner. THF-mediated inhibition on proinflammatory cytokine release is probably associated with downregulation of LPS-induced TLR4 mRNA augmentation. *In vivo*, THF could significantly protect mice against a lethal challenge with heat-killed *E. coli* 35218 (*E. coli* 35218) in a dose-dependent manner, and decreased the plasma LPS level in endotoxemia mice. These findings provide compelling evidence that THF may be an important potential drug for sepsis treatment. Considering the inhibitory effects of THF on LPS-induced cytokine release are unlikely due to its nonspecific cellular toxicity, THF should be considered as a safe putative candidate for development as a drug for sepsis treatment.


## 1. Introduction

Sepsis results in the activation of numerous proinflammatory mediators such as TNF-α, IL-6 and IL-12. This condition may result in multiple organ dysfunction syndrome (MODS), septic shock and ultimately death [1,2]. An epidemiological study found that the incidence of sepsis was approximately three cases per 1000 people, and the morbidity is increasing in recent years. The overall mortality rate is about 30%, rising to 40% in the elderly and more than 50% in patients with septic shock [3].

Lipopolysaccharide (LPS/endotoxin), the major constituent of the outer membrane of gram-negative bacteria, is a common trigger of sepsis. The overproduction of LPS-induced cytokines such as TNF-α, IL-6 and IL-12, has been considered central to the pathophysiologic derangement associated with sepsis and septic shock. Despite improved care, the hospital mortality rate from severe sepsis and septic shock has not improved significantly over recent decades [4]. There are currently few effective adjuvant therapies in clinical use except activated protein C, which targets the coagulation system [5–7]. Therefore, it is important to investigate new anti-LPS drugs to potentially identify a clinically relevant anti-sepsis drug.

Lipid A, an evolutionarily conserved region of LPS, has been identified as the toxic component of LPS and hence represents an ideal target for anti-sepsis drug development. The mechanism of biological activity of LPS probably involves in specific binding of lipid A moiety to the cationic residues of a receptor molecule. Several agents such as polymyxin B (PMB), bactericidal/permeability increasing protein (BPI) and Limulus anti-LPS factor (LALF) bind to lipid A and antagonize the effects of LPS exhibit extensive physicochemical properties (i.e., hydrophobicity and cationic charge) within their binding domains [8].

Medicinal plants have been used as traditional remedies for hundreds of years in China. Recently, clinical trials have shown a lot of traditional Chinese herbs possess anti-sepsis function [9]. Therefore, it is promising to explore anti-sepsis drug form these herbs. However, these herbs usually have a large number of complex constituents, which extremely difficult to identify the anti-sepsis component, which limit their clinical uses. Therefore, the platform to screen anti-sepsis component from traditional Chinese herbs was established using biosensor affinity technology in 2004, and an anti-sepsis

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monomer, 1, 2, 3, 4, 6-β-D-pentagalloylglucose (PGG), was obtained using silica gel chromatography and HPLC [10].

In present experiments, we describe the screening and isolation of anti-sepsis monomer from traditional Chinese herbs using affinity technology. An anti-sepsis monomer was isolated from *S. baicalensis* Georgi (Huang Qin) and its anti-sepsis activity was investigated *in vitro* and *in vivo*.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Reagents

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). Macroporous adsorptive resins AB-8 was purchased from Qingdao Marine Chemical Factory (Qingdao, China). Mouse TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biosource International (Camarillo, CA, USA). The kinetic turbidimetric *Limulus* amebocyte lysate (LAL) kit was purchased from Zhanjiang A & C Biological Ltd. (Zhanjiang, China). The RNA easy kit (ReverTra Ace$^{-\alpha-}$) and Real-time PCR Master Mix (SYBR Green) were obtained from Toyoboco Ltd. (Osaka, Japan).

#### 2.1.2. Traditional Chinese herbs

Twelve traditional Chinese herbs were purchased from Sichuan Province, and identified in the Chongqing Academy of the Chinese Materia Medica (Chongqing, China).

#### 2.1.3. Animals

One hundred and twenty 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 mice on the day of the experiments was (20.6±1.5) 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 strains

Bacterial strains of *E. coli* ATCC 35218 were maintained in our laboratory and used for the mouse sepsis model. Single colonies from viable, growing Luria–Bertani (LB) agar plates were transferred to sterile liquid LB medium (containing 10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter) and cultivated aerobically in 50-ml volumes at 37 °C in a heated, 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 in sterile NS to achieve a concentration of approximately $1\times10^{10}$ colony-formation units (CFU) per milliliter. Finally, the bacterial suspensions were incubated in a water bath at 100 °C for 30 min in order to inactivate the cells [11].

#### 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. In each experiment, $10^6$ cells/ml were used, except where otherwise indicated.

### 2.2. Methods

#### 2.2.1. Screening and isolation of lipid A-binding components/monomer from Chinese herbs

*2.2.1.1. Screening of lipid A-binding herbs using biosensor technology.* Lipid A was immobilized on the surface of a hydrophobic cuvette according to the manufacturer's instructions (Thermo Labsystem, USA), and as described previously [12]. Twelve herbs were each soaked in water for 24 h, after washing thoroughly with distilled water, and then boiled in water at 100 °C for 45 min. After filtration, the material was centrifuged at 4000 ×*g* for 30 min and the supernatants collected, and are termed here "aqueous extract". Five microlitres of aqueous extract (15 g/L) from each herb 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).

*2.2.1.2. Isolation lipid A-binding components from S. baicalensis Georgi using macroporous adsorptive resins technology.* An aqueous extract from *S. baicalensis* Georgi was subjected to column chromatography on macroporous adsorptive resins AB-8 (5 cm i.d.×60 cm) eluted with distilled water and gradient ethanol (10%, 20%, 40% and 60%). The elute was collected and concentrated by rotary-evaporation (BUCHI Rotavapor R205, Switzerland), respectively, to yield a total five fraction and named SbG-1~5. Five microlitres of SbG-1~5 (2.0 g/L) was assayed for its binding activity to lipid A in turn as described as above, Thus, the compound (SbG-4) which has the highest binding activity to lipid A was screened among them.

*2.2.1.3. Isolation lipid A-binding monomer from S. baicalensis Georgi using HPLC technology.* The fraction (SbG-4) screened in experiment 2.2.1.2 was injected onto HPLC system (Agilent, USA) eluted with methanol/0.5% acetic acid (20:80 to 60:40 gradient) to yield a total five HPLC fraction and named 5KL-1–5. Five microlitres of 5KL-1–5 (1.0 g/L) was assayed for its binding activity to lipid A as described as above. The fraction (5KL-1) which has the highest binding activity to lipid A was screened among them and further purified by HPLC. Thus, a HPLC purified product with lipid A-binding activity was obtained. Furthermore, purity factor calculation was performed for its peak purity evaluation as described in Agilent operating manual. After analyzed by mass spectrometry, infrared and NMR (nuclear magnetic resonance) at the National Center of Biomedical Analysis (Beijing, China), a monomer was harvested.

#### 2.2.2. In vitro studies

*2.2.2.1. Neutralization of endotoxin.* The ability of the monomer to neutralize LPS was assayed using the LAL test, which is an extremely sensitive indicator of the presence of free, non-neutralized LPS [9]. Different concentrations of the monomer (0–4.0 mg/L) were incubated with LPS (0.1 μg/L) 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).

*2.2.2.2. Cytokine release induced by LPS.* 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. The cells were pretreated with the monomer at the indicated dose (0, 10, 20, 40, 80, 160 mg/L) for 30 min, and then stimulated with LPS (100 μg/L) for 4 h and 12 h. The supernatants were collected to assess TNF-α and IL-6 levels using the appropriate ELISA kits, respectively.

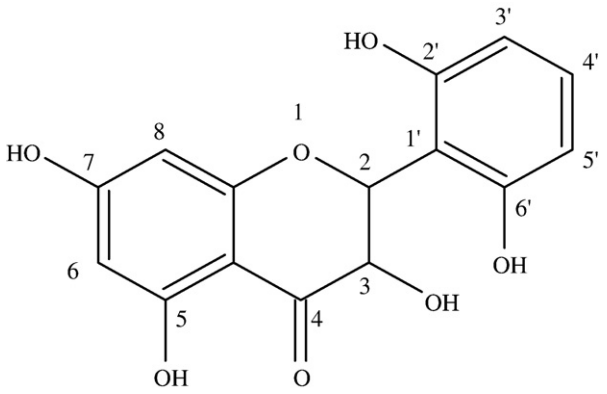


**Fig. 1.** Structure of 2′,5, 6′,7-tetrahydroxyflavanonol (THF).

*2.2.2.3. TLR4 and TNF-α mRNA expression.* The RAW264.7 cells (3.0 ml) were grown in 6-well polystyrene plates and incubated with different concentrations (0, 40, 80 and 160 mg/L) of the monomer for 30 min before the addition of LPS (100 μg/L) for 4 h to extract the RNA. After removing the medium, cells were washed once with sterile PBS. The RNA from the cells was extracted using the RNA easy kit according to the manufacturer's instructions (ReverTra Ace$^{-\alpha-}$). After DNase I treatment, 2 μg of RNA was reverse transcribed with AMV reverse transcriptase. A SYBR Green Real-time PCR Master Mix 10 μl, distilled water 8.4 μl and cDNA 1 μl reaction mixture was distributed into different PCR tubes. Forward and reverse primers (0.3 μl/each) corresponding to different individual genes were added to the PCR tubes (to select β-actin as internal control), and subjected to real-time fluorogenic PCR. The reactions conditions as follow: 1) denature at 94 °C for 300 s (1 cycle), 2) denature at 94 °C for 30 s→anneal at 59 °C for 30 s→extend at 72 °C for 45 s (40 cycles). After the PCR is finished, perform dissociation curve analysis to see if there is any bimodal dissociation curve or abnormal amplification plot. For each sample, mRNA expression levels for specific transcripts were normalized to the amount of β-actin and the $2^{-\Delta\Delta CT}$ method was used to analyze the gene expression data as described by Livak et al. [13]. The following primers were used: TLR4 (132-bp product), forward (5′-AG GCA TGG CAT GGC TTA CAC-3′) and reverse (5′-GGC CAA TTT TGT CTC CAC AGC-3′); TNF-α (217-bp product), forward (5′-CAG GTT CTG TCC CTT TCA CTC ACT-3′) and reverse (5′-GTT CAG TAG ACA GAA GAG CGT GGT-3′); mouse β-actin (191-bp product), forward (5′-GGG AAA TCG TGC GTG ACA TCA AAG-3′) and reverse (5′-CAT ACC CAA GAA GGA A GG CTG GAA-3′).

*2.2.2.4. Cytotoxicity assay.* Cytotoxicity was established using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (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 the monomer for 24 h. Subsequently, 20 μl of MTT solution (5 g/L in PBS) were added in a total volume of 200 μl medium. After cells were incubated for 4 h at 37 °C and 5% CO2, 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).

*2.2.3. In vivo studies*

*2.2.3.1. Experimental sepsis animal models and monomer treatment in mice.* One hundred of mice were randomly divided into five groups (20 mice/group) and were intravenously injected as follows: heat-killed *E. coli* ($1.30\times10^{11}$ CFU/kg) alone in group A, heat-killed *E. coli* ($1.30\times10^{11}$ CFU/kg) and immediate subsequent injection of monomer (10 mg/kg) in group B, heat-killed *E. coli* ($1.30\times10^{11}$ CFU/kg) and immediate subsequent injection of monomer (20 mg/kg) in group C, heat-killed *E. coli* ($1.30\times10^{11}$ CFU/kg) and immediate subsequent injection of monomer (40 mg/kg) in group D, and the monomer (100 mg/kg) alone in group E. 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. Plasma LPS level in endotoxemia mice.* Twenty KM mice were randomly divided into five groups (4 mice/group). Group 1 was given LPS (50 μg/kg), group 2, 3 and 4 were respectively given 10, 20 and 40 mg/kg of the monomer followed by injection with LPS (50 μg/kg), and group 5 was injected with saline as a negative control. 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 plasma was diluted in 380 μl of saline. The endotoxin level in the diluents was assayed by the LAL test.

*2.2.4. Statistics and presentation of data*

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. Each experiment was repeated at least twice and each data point represents the mean of at least three parallel samples. A Student's *t*-test was used to examine the differences between groups. A (double-sided) *p*-value less than 0.05 was considered significant, and a *p*-value less than 0.01 was considered very significant.

## 3. Results

*3.1. S. baicalensis Georgi was screened form twelve herbs*

Among twelve Chinese herbs (*Radix Zanthoxyli, Fructus Forsythiae, Radix Bupleuri, Cortex Phellodendri, Tripterygium wilfordii, Radix et Rhizoma Rhei, Rhizoma Coptidis, Flos Lonicerae, Radix Salviae Miltiorrhiae, Scutellaria baicalensis Georgi, Herba Houttuyniae, Radix Isatidis and Radix et Rhizoma Rhei*) were found to possess higher lipid A-binding activities (RU>100 arc sec) (Supplementary Fig. 1). Because the gelatin test showed *Radix et Rhizoma Rhei* was strong positive (data not shown), it was supposed to contain more tannin compounds in *Radix et Rhizoma Rhei*. Since tannin compounds could interfere with isolation of subsequent isolation of monomers, we selected *S. baicalensis* Georgi in subsequent experiments.

*3.2. A lipid A-binding fraction was extracted from S. baicalensis Georgi*

Five fractions were isolated from *S. baicalensis* Georgi by macroporous adsorptive resins AB-8 and named SbG-1, -2, -3, -4, -5 in turn,

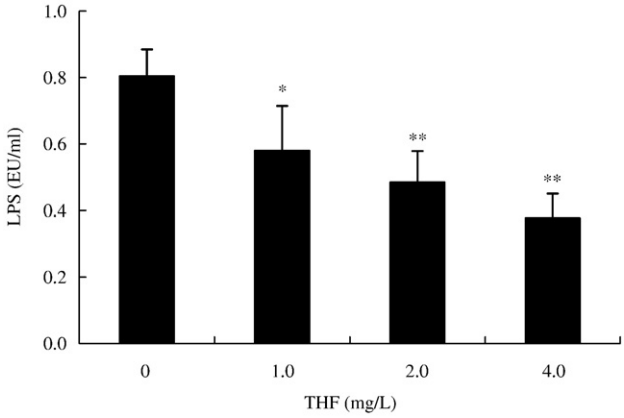


**Fig. 2.** Neutralization of THF to LPS *in vitro*. Indicated concentrations of THF were incubated with 0.1 μg/L LPS at 37 °C for 30 min. Subsequently, 100 μl of this mixture was added to an equal volume of the LAL reagent and the kinetic turbidity was measured. The test was repeated three times. *$p<0.05$ and **$p<0.01$ as compared to the treatment without THF.

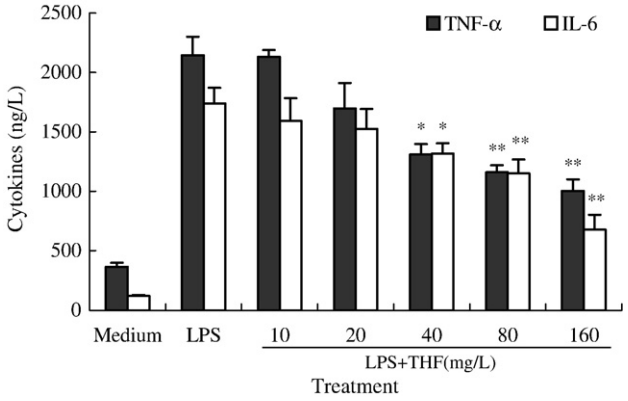


**Fig. 3.** Effect of THF on cytokines release from RAW264.7 cells induced by LPS. RAW264.7 cells (0.2 ml) was pre-incubated with indicated concentrations of THF for 30 min, then stimulated with LPS (100 μg/L). After cells were incubated for another 4 h and 12 h, the levels of TNF-α and IL-6 in the supernatants were analyzed using the appropriate ELISA kits, respectively. Cytokine levels were expressed as mean±standard in ng/L. Student's *t*-test was used to examine the differences. *$p<0.05$ and **$p<0.01$ compared to only LPS treatment.

their binding activities to lipid A were detected using biosensor method. Among them, the binding activities of SbG-4 and SbG-5 to lipid A (their RU value was 113.4 arc sec and 73.8 arc sec, respectively) were higher than others (Supplementary Fig. 2). Because of poor water-solubility of SbG-5, SbG-4 was further isolated by HPLC.

### 3.3. 2′,5,6′,7-tetrahydroxyflavanonol (THF) was isolated from SbG-4

SbG-4 was subjected to HPLC eluted with methanol/0.5% acetic acid to yield a total five HPLC fractions, and named 5KL-1, -2, -3, -4, -5 (Supplementary Fig. 3). Among them, the lipid A-binding activity of 5KL-1 was the highest (Supplementary Fig. 4). Thus, we selected 5KL-1 to further purify monomer in later experiments.

Since there was only a single peak in the purified product of 5KL-1 using HPLC chromatographic analysis (Supplementary Fig. 5), we collected this peak and its purity factor is 999.685 which was set according to Agilent's define (Supplementary Fig. 6). After its structure was analyzed by mass spectrometry, infrared and NMR (nuclear magnetic resonance) at the National Center of Biomedical Analysis (Beijing, China) (data not shown), The monomer with binding activity for lipid A was confirmed as 2′,5,6′,7-tetrahydroxyflavanonol (THF), its molecular formula was $C_{15}H_{12}O_7$ (Fig. 1).

### 3.4. THF directly neutralizes LPS in vitro

As shown in Fig. 2, THF in 1.0, 2.0 and 4.0 mg/L, neutralized 27.9%, 39.8% and 53.1% of LPS in 0.1 μg/L, respectively. With concentration increasing, the neutralizing effect of THF on LPS enhanced, which indicated THF could directly neutralize LPS *in vitro*.

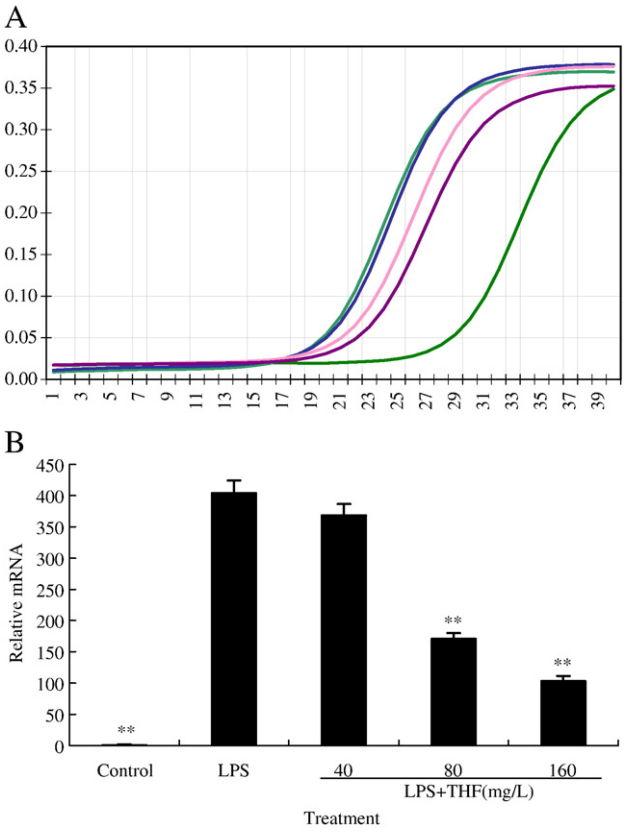


**Fig. 4.** Effect of THF on the stimulation of mRNA expression of TNF-α in RAW264.7 cells by LPS. The RAW264.7 cells (3.0 ml) were incubated with THF at a gradient concentrations (0, 40, 80, 160 mg/L) for 30 min in 6-well polystyrene plates and then cultured with LPS (100 μg/L) for 4 h. The amplification plot of real-time quantitative PCR was shown in (A), the reaction curves illustrated PCR proceeding of different samples, from left to right: LPS treatment, THF 40, 80, 160 mg/L treatment and control. Meanwhile, the fold changes of TNF-α mRNA were analyzed by using the $2^{-\Delta\Delta CT}$ method (B). Values are expressed as the mean±standard of triplicate tests, *$p<0.05$ and **$p<0.01$ as compared to only LPS treatment.

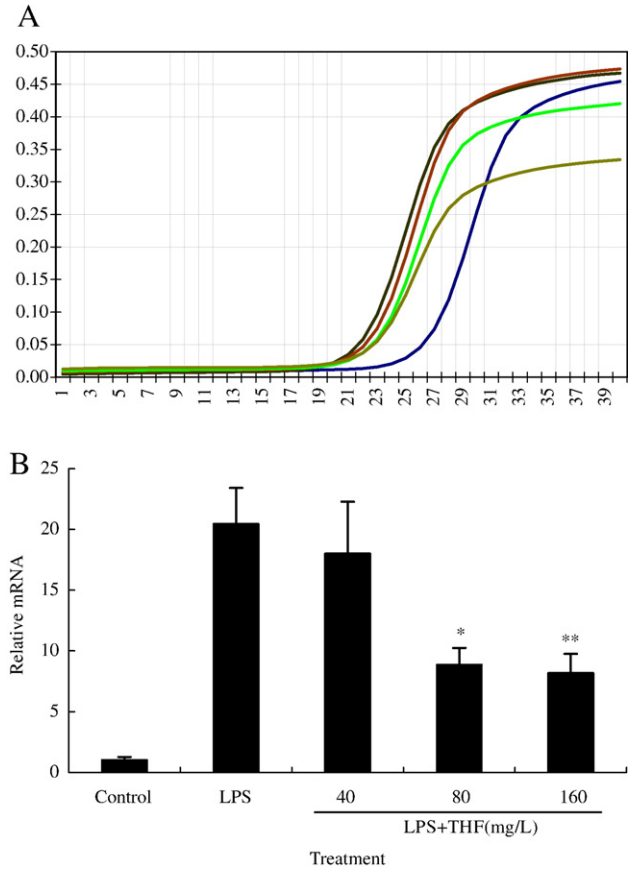


**Fig. 5.** Effect of THF on the stimulation of mRNA expression of TLR4 in RAW264.7 cells by LPS. The RAW264.7 cells (3.0 ml) were incubated with THF at a gradient concentrations (0, 40, 80, 160 mg /L) for 30 min in 6-well polystyrene plates and then cultured with LPS (100 μg/L) for 4 h. The amplification plot of real-time quantitative PCR was shown in (A), the reaction curves illustrated PCR proceeding of different samples, from left to right: LPS treatment, THF 40, 80, 160 mg/L treatment and control. Meanwhile, the fold changes of TLR4 mRNA were analyzed by using the $2^{-\Delta\Delta CT}$ method (B). Values are expressed as the mean±standard of triplicate tests, *$p<0.05$ and **$p<0.01$ as compared to only LPS treatment.

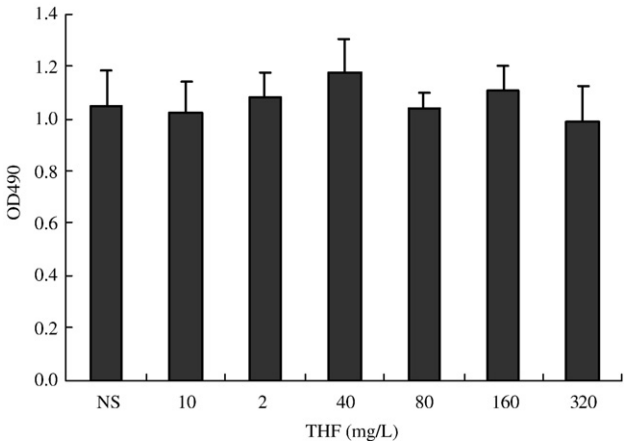


**Fig. 6.** Cytotoxicity of THF on RAW264.7 cells. RAW264.7 cells were plated in a 96-well plate in DMEM. After an overnight culture period, cells were washed with DMEM and incubated NS or various concentrations of the THF for 24 h. Subsequently, 20 μl of MTT solution (5 g/L in PBS) were added to the medium in a total volume of 200 μl. After the cells were incubated for 4 h at 37 °C and 5% CO2, the supernatant was removed and 150 μl DMSO was added to each well to dissolve the produced formazan crystals. The extinction was measured at 490 nm using a microplate reader. Compared to the NS treatment, the $OD_{490}$ values of various concentrations of the THF treatment weren't significant difference.

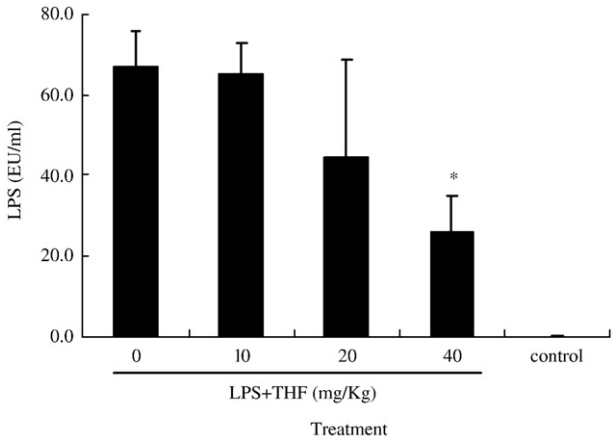


**Fig. 8.** THF neutralizes LPS activity in endotoxemia mice. Twenty mice were randomly divided into five groups (4 mice/group). Group 1 was given 50 μg/kg LPS; groups 2, 3 and 4 were given 10, 20 and 40 mg/kg THF, respectively, followed by infusion with LPS and group 5 was infused with saline as a negative control, 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 plasma 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. *$p<0.05$ as compared to the treatment without THF.

### *3.5. THF inhibits TNF-α mRNA expression, and TNF-α and IL-6 release from RAW264.7 cells*

Cytokines such as TNF-α and IL-6 plays a key role in sepsis [14]. In order to confirm whether THF's neutralizing effect of on LPS was paralleled with an inhibiting effect on cytokine release induced by LPS, the ability of THF to inhibit TNF-α and IL-6 secretion in LPS-stimulated RAW264.7 cells was determined. As shown in Fig. 3, LPS could induce RAW264.7 cells to release TNF-α and IL-6. However, pre-treatment of cells with THF (80–160 mg/L) significantly inhibited cytokine release induced by LPS in a dose-dependent manner. In addition, we also found THF down-regulated TNF-α mRNA expression in LPS-stimulated RAW264.7 cells using real-time quantitative PCR method (Fig. 4).

### *3.6. THF reduces TLR4 mRNA expression*

TLR4 signaling is required for the induction of cytokine release by LPS [15–17]. To investigate whether THF could affect TLR4 signaling, the expression of TLR4 mRNA was observed using real-time quantitative PCR method. As shown in Fig. 5, TLR4 mRNA was expressed at a very low level in non-stimulated cells. But LPS markedly up-regulated TLR4 mRNA expression. Significantly, THF (80, 160 mg/L) could down-regulated the up-regulation of TLR4 mRNA ($p<0.01$).

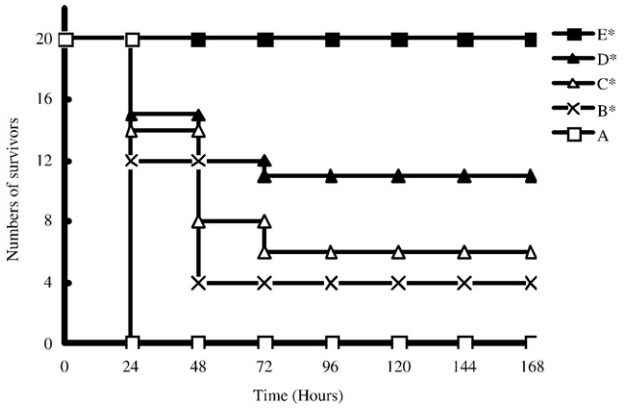


**Fig. 7.** Effect of THF on the survival rate of mice challenged with a lethal dose heat-killed *E. coli*. Mice were randomly divided into five groups ($n=20$). Groups of mice were treated with □/A, heat-killed *E. coli* ($1.30\times10^{11}$ CFU/kg) alone; x/B, THF(10 mg/kg) and heat-killed *E. coli*; Δ/C, THF (20 mg/kg) and heat-killed *E. coli*; ▲/D, THF (40 mg/kg) and heat-killed *E. coli* and ■/E, THF (100 mg/kg) alone. The mice were observed for 7 days. *$p<0.01$ as compared to the heat-killed *E. coli* group.

### *3.7. THF has no cellular toxicity in vitro*

To exclude the possibility of the monomer affecting the cell viability, the cytotoxic effects of THF on RAW264.7 cells were investigated using the MTT assay. THF (10–320 mg/L) treatment for 24 h did not produce cellular toxicity in RAW264.7 cells (Fig. 6). Since the concentration of THF used was less than 320 mg/L, and incubation time was less than 24 h in *in vitro* experiments, so there is no correlation between the THF-induced inhibition on cytokine release and its cytotoxicity.

### *3.8. THF protects mice against a lethal dose heat-killed E. coli challenge*

To test whether THF could protect mice from a lethal challenge with heat-killed *E. coli*, test animals were injected with different concentrations of THF, prior to a lethal challenge with heat-killed *E. coli* ($1.30\times10^{11}$ CFU/kg, i.v.). All mice challenged by heat-killed *E. coli* (without THF pre-treatment), all died within 24 h. By contrast, animals pretreated with THF were protected from heat-killed *E. coli*-induced lethality, in a dose-dependent manner; PGG (40 mg/kg) protected mice to a significant degree ($p<0.05$) [10] and THF (40 mg/kg) had a highly significant protective effect ($p<0.01$) (Fig. 7).

### *3.9. THF neutralizes LPS activity in endotoxemia mice*

In order to test whether THF could neutralize LPS activity *in vivo*, the LPS concentration in the endotoxemia mice treated with or without THF were detected. As shown in Fig. 8, the LPS level increased sharply in the mice challenged with LPS, but more than 20, 40 mg/kg of THF decreased LPS level. Especially, 40 mg/kg of THF significantly decreased LPS level ($p<0.05$).

## 4. Discussion

In this study, we reported that an anti-sepsis monomer, 2′, 5, 6′, 7-tetrahydroxyflavanonol (THF) was isolated from *S. baicalensis* Georgi

(Huang Qin) under the direct of bioactivities. *In vitro*, THF could neutralize LPS and inhibit proinflammatory cytokines release from RAW264.7 cells induced by LPS. *In vivo*, THF could protect mice against a lethal challenge with heat-killed *E. coli* and significantly decrease the plasma LPS levels in endotoxemia mice. To the best of our knowledge, this is the first report to demonstrate the anti-sepsis activity of THF.

*S. baicalensis* Georgi (Huang Qin) is one of the most widely used in traditional Chinese medicine. The dry roots of *S. baicalensis* Georgi, officially listed in the Chinese Pharmacopoeia, have been used for anti-inflammation, anticancer, antiviral and antibacterial infections of the respiratory and the gastrointestinal tract, reducing the total cholesterol level and decreasing blood pressures [18]. Flavonoid such as baicalin, baicalein, wogonin and oroxylin A was the main active component, but its bioactivities were not completely clear. Baicalein was found as a novel class of natural free radical scavenger of flavonoid [19]. It could protect neuronal cells from oxidative stress by scavenging free radicals [20], and exhibit potent protective effect against endotoxic shock in rats *in vivo* and *in vitro* [21–22]. The mechanism of the antiinflammatory action of baicalein and wogonin were related to attenuation of LPS-stimulated nitric oxide synthase in macrophages by inhibition of the activation of NF-κB [21–22]

In present experiments, we isolated THF under the direction of bioactivity although THF was not first isolated in our lab. But we firstly discovered its anti-sepsis bioactivities since it was first isolated from *S. baicalensis* Georgi in 1981 [23].

Previously we immobilized lipid A on the hydrophobic cuvette of biosensor as the target to screen lipid A-binding components/monomer from traditional Chinese herbs [10]. PGG with anti-sepsis bioactivity was obtained using silica gel chromatography and HPLC. Since PGG chemically belongs to tannic acid, tannic acid is unsuitable for drug. Therefore, under the direction of neutralization of LPS and reducing proinflammatory cytokines, in the present experiment, we got THF belonging to flavonoid. These results showed it was effective and feasible to get new compound or find new bioactivities from traditional Chinese herbs and other plants using our established experimental platform.

LPS is a common trigger of sepsis. Therefore, one of several adjuvant therapeutic approaches for severe sepsis is currently focusing on the neutralization of LPS. Our data demonstrated that THF not only directly bound to LPS, but also neutralized LPS *in vitro* and decreased the plasma LPS level *in vivo*. Usually, agents with positive charge such as PMB and BPI could bind to LPS with large negative charge, and then lead to the loss of LPS activity [12]. Interestingly, the structure of THF can't form positive charge unlike PMB and BPI. Therefore, how THF to interact with LPS is unclear.

Proinflammatory such as TNF-α, IL-6 and IL-12 induced by LPS lead to the pathophysiologic derangement associated with sepsis and septic shock [24]. TNF-α was thought to be an early cytokine, whereas both IL-6 and IL-12 were considered later cytokines [13,25]. Our data demonstrated that THF not only down-regulated TNF-α mRNA expression but also decreased TNF-α and IL-6 release from RAW264.7 cells induced by LPS in a dose-dependent manner.

TLR4 is a pattern recognition receptor for LPS. LPS activates the TLR4-mediated signal transduction pathway to regulate the release of cytokines [16]. Our data demonstrated that THF could down-regulated TLR4 mRNA expression in RAW264.7 cells induced by LPS in a dose-dependent manner. THF-mediated inhibition on proinflammatory cytokine release is probably associated with downregulation of LPS-induced TLR4 mRNA augmentation.

The inhibitory effects of THF on LPS-induced cytokine release are unlikely due to its nonspecific cellular toxicity. The concentration of THF used in the present experiments did not affect RAW264.7 cell viability. More importantly, in mice treated with THF (100 mg/kg), we did not observe any side effects, such as liver or kidney dysfunction (data not shown). Therefore, THF should be considered as a safe putative candidate for development as a drug for sepsis treatment.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.intimp.2008.07.017.

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