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Targeting CpG DNA to screen and isolate anti-sepsis fraction and monomers from traditional Chinese herbs using affinity biosensor technology

Xin Liu [a,1], Juan Cheng [b,1,2], Xinchuang Zheng [a], Yiguo Chen [a], Chong Wu [b,2], Bin Li [b,2], Jianfeng Fu [a], Hongwei Cao [a], Yongling Lu [a], Jun Li [b,2], Jiang Zheng [a,*], Hong Zhou [b,*]

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

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ABSTRACT

Bacterial DNA/CpG DNA is recognized as a key molecule during the pathogenesis of sepsis. Therefore, preventing CpG DNA from binding to its receptor is considered as the most promising strategy. In the present experiments, *Radix et Rhizoma Rhei* had the highest CpG DNA-binding ability among the seventy-eight traditional Chinese herbs. After the isolation of silica gel chromatography and high performance liquid chromatography (HPLC) and evaluation with affinity biosensor, the active fraction was confirmed and named Fraction D. It was found that *in vitro*, Fraction D bound to both CpG DNA and lipid A with high affinity, and strongly inhibited LPS- and CpG DNA-induced TNF-α release from RAW264.7 cells in a dose-dependent manner. Furthermore, Fraction D reduced the expression of TLR9 mRNA up-regulated by CpG DNA. *In vivo*, Fraction D protected mice challenged with lethal heat-killed *E. coli*. Using HPLC method, two monomers with high affinity for CpG DNA were isolated and identified as rhein and emodin. Rhein could significantly reduce CpG DNA- and LPS-induced TNF-α release, but emodin only reduced CpG DNA-induced TNF-α release. Rhein in combination with emodin could play synergistic inhibitory effect on both CpG DNA and LPS-induced TNF-α release, which contributed to the bioactivity of Fraction D. In conclusion, we successfully established the platform to screen anti-CpG DNA components of traditional Chinese herbs using affinity biosensor technology, got active Fraction D from *Radix et Rhizoma Rhei* and determined rhein and emodin as the main bioactive ingredients in Fraction D.

1. Introduction

Sepsis is a generalized inflammatory response to an infection. Severe sepsis results in multi-organ dysfunction, septic shock and death. Recent surveys conducted in the U.S. and in Europe have indicated that approximately 2%–11% of all hospital and intensive care unit admissions can be attributed to severe sepsis. Despite improvements in supportive care and the increased availability of effective antibacterial agents, hospital mortality rates from severe sepsis and septic shock (50%–60%) have not improved over recent decades [1,2]. Unfortunately, many experimental inflammatory antagonist-based therapies have failed in sepsis trials, and currently there is only one adjuvant therapy in clinical use, e.g. activated protein C, which targets the coagulation system [3]. 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 [4,5]. Thus it is important to investigate additional inflammatory antagonist-based treatments with the aim of developing a clinically effective anti-sepsis drug.

Sepsis is triggered by the presence of invasive bacteria and bacterial components, such as bacterial DNA (bDNA) and lipopolysaccharide (LPS [endotoxin]) [6,7]. Synthetic oligodeoxynucleotides containing CpG motifs (CpG DNA) mimic the activity of bDNA. Excessive bDNA and CpG DNA-driven immune activation may induce hyperinflammatory responses, even systemic inflammatory response syndrome (SIRS) and sepsis [7]. It is therefore necessary to develop treatments, which can be used to balance the activity of CpG DNA and reduce the release of cytokines induced by CpG DNA exposure. Previously, some neutralizing or suppressive CpG ODNs (CpG-N ODNs) were developed to inhibit bioactivities of bDNA [8,9]. In our lab, chloroquine, an acidic inhibitor of endosome, was discovered to inhibit proinflammatory cytokines release induced by bDNA *in vitro* and *in vivo* [10].

* Corresponding authors. Zheng is to be contacted at Medical Research Center, Southwestern Hospital, The Third Military Medical University, Gaotanyan Street 30, Shapingba District, 400038, Chongqing 400038, PR China. Tel.: +86 23 68754435; fax: +86 23 65460584. Zhou, Department of Pharmacology, College of Medicine, The Third Military Medical University, Gaotanyan Street 30, Shapingba District, Chongqing 400038, PR China. Tel./fax: +86 23 68752266.
E-mail addresses: zhengj@mail.tmmu.com.cn (J. Zheng), zhouh64@mail.tmmu.com.cn (H. Zhou).
[1] They equally contributed to this work.
[2] Tel./fax: +86 23 68752266.

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In 2002, we successfully established an effective method using affinity biosensor technology to discover agents to neutralize LPS from traditional Chinese herbs [11]. Now, we wonder to know whether there are anti-CpG DNA components in traditional Chinese herbs. With these considerations in mind, we undertook the current study to investigate effective anti-CpG DNA agents from the traditional Chinese herbs using affinity biosensor technology. The fraction from *Radix et Rhizoma Rhei* was isolated, and its active fraction was investigated both *in vitro* and *in vivo*. Further, the main ingredients were isolated and identified; their bioactivities were investigated *in vitro*.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

CpG DNA 1826 (5′-TCCATGACGTTCCTGATGCT-3′, which is the optimal murine sequence) and 5′-biotin-labeled CpG DNA 107 (5′ TGGCGCGGGGCG-3′, which is the optimal human sequence) with a nuclease-resistant phosphorothioate backbone were synthesized by SBS Genetech (Beijing, China).

LPS (from *Escherichia coli* O111:B4), lipid A (from Samonella Re595) and polymyxin B sulfate salt (PMB) were purchased from Sigma Chemicals (St Louis, MO, USA). Dexamethasone sodium phosphate injection (DMX) was purchased from Hubei Tianyao Pharmaceutical Group Company Limited (Hubei, China). Standard substances rhein and emodin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Silica gel was from Qingdao Marine Chemical Factory (Qingdao, China). Mouse TNF-α ELISA kit was purchased from Biosource International (Camarillo, CA, USA) and Diaclone Research (Besançon, France), respectively.

2.1.2. Traditional Chinese herbs

Seventy-eight traditional Chinese herbs (see Table 1) 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 ten Kunming mice (4–6 weeks old) were obtained from the Experimental Animal Center of the Chongqing Medical University (Chongqing, China). Equal numbers of male and female mice were used. The weight of the mice on the day of the experiments was 17.8 ± 2.0 g.

2.1.4. Cell culture

The murine macrophage-like cell line, RAW264.7 (American Type Culture Collection, Manassas, VA), was cultured at 37 °C in a 5% CO_2 humidified incubator and maintained in DMEM medium supplemented with 10% low-endotoxin fetal calf serum (Hyclone, Logan, UT, USA), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were diluted with 0.4% trypan blue in PBS (0.1 mM, pH 7.2) and live cells were counted with a hemacytometer.

2.2. Methods

2.2.1. Preparation of bacterial strain

Bacterial strains of *E. coli* ATCC 35218 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 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 re-suspended and diluted into sterile saline to achieve a concentration of approximately 1×10^{10} colony formation units (CFU)/ml. Finally, bacterial suspensions were incubated in a water bath at 100 °C for 30 min in order to kill the bacteria.

Table 1
List of the traditional Chinese herbs examined in this study.

Name		
Aconitum carmichaeli Debx	Fructus Mume	Radix Polygoni Multiflori
Belamcanda chinensis	Herba Andrographitis	Radix Rubiae
Bulbus Fritillariae irrhosae	Herba Artemisiae Annuae	Radix Puerariae Lobatae
Centiana Scabra Bge	Herba Commelinae	Radix Pulsatillae
Calvatia Lilacina	Herba Equiseti Hiemalis	Radix Salviae Miltiorrhiizae
Calvatia Gigantea Lbyd	Herba Hedyotidis	*Radix Sanguisorbae*
Caulis Sargentodoxae	Herba Portulacae	Radix Scutelariae
Chinese Lobelia Herb	Herba Saussureae	Radix Sophorae Flavescentis
Chinese White Olive	Herba Schizonepetae	Radix Scrophulariae
Colla Corii Asini	Herba Sedi	Radix Stemmacanthae Uniflori
Cortex fraxini	*Hibiscus mutabilis* L	Radix Trichosanthis
Cortex Phellodendri	*Houttaynia cordata* Thunb	Rhizoma Anemarrhenae
Cortex Eucommiae	Cacumen Platycladi	Rhizoma Cimicifugae Foetide
Cortex Cinnamomi	*Iphigenia indica* Kunthet	Rhizoma Coptidis
Densefruit Pittany Root-Bark	*Mentha haplocalyx* Briq	Rhizoma Bistortae
Flos Lonicerae	Monimopetalnm Chinese	Rhizoma Bletillae
Folium Isatidis	Peony Root	Rhizoma *Dioscoreae bulbifera*
Folium Sennae	*Poria cocos* Wolf	Rhizoma Phragmitis
Flos Sophorae	Radix Ampelopsis	Rhizoma Smilacis Glabrae
Fructus Arctii	Radix Bupleuri	Semen Persicae
Fructus Aurantii Immaturus	Radix Cynanchi Atrati	Spica Prunellae
Fructus Bruceae	Radix et Rhizoma Tripterygii	Spohora Tonkinnesis Gapnep
Fructus Crataegi	*Radix et Rhizoma Rhei*	Viola Yedoensis Makino
Fructus Cnidii	Radix Isatidis	Whiteflower Patrinia Herb
Fruetus Forsythiae	Radix Phytolaccae	Fructus Crataegi
Fructus Gardeniae	Radix Platycodi	Stings of *Gleditsia inensis*

2.2.2. Preparation of aqueous extractions of traditional Chinese herbs

2.2.2.1. Preparations of water extractions for affinity detection. Crude drugs of seventy-eight herbs were pulverized. One gram of each powder was added with 10 ml distilled water, soaked for 2 h, and then boiled at 100 °C for 45 min. After filtration, the material was centrifuged at 4000 ×*g* for 30 min and the supernatants, termed here as "aqueous extractions", were collected for affinity detection.

2.2.2.2. Preparations of water extractions of Radix et Rhizoma Rhei Palmati for further isolation of monomers. Five kilograms of *Radix et Rhizoma Rhei Palmati* were washed with distilled water, dried and pulverized. The powder was mixed with 50,000 ml distilled water and soaked for 2 h, and then boiled at 100 °C for 45 min. The mixture was filtrated and centrifuged at 4000 ×*g* for 30 min. the supernatants were collected, concentrated by rotary-evaporation and freeze dried.

2.2.3. Immobilization of Lipid A or CpG DNA on cuvette

Lipid A Re595 from *E. coli* was immobilized on the surface of a hydrophobic cuvette according to the manufacturer's instructions (Thermo Labsystem, USA), and as described previously [11]. Briefly, hydrophobic cuvette was inserted in the instrument and washed five times with 50 μl 2-propanol. Data acquisition initiated with 1 min acquisition of baseline data. Then the cuvette was wash seven times with 60 μl PBS/AE (phosphate buffered saline containing 0.025% (w/v) sodium azide and 1 mM EDTA, pH 7.4) and 10 min of baseline data was collected. The cuvette was further washed seven times with 50 μl isopropanol, added with 20 μl lipid A (2 mg/ml in chloroform), and collected about 1 min of binding data. Then it was

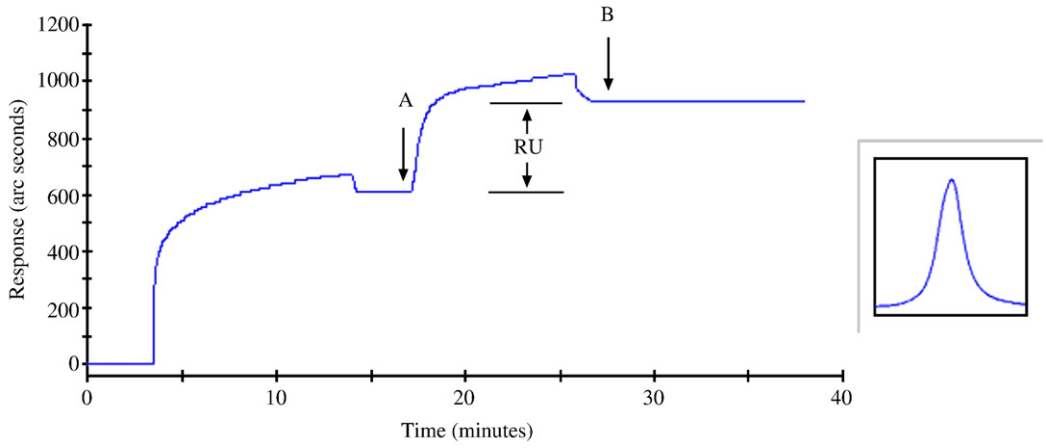
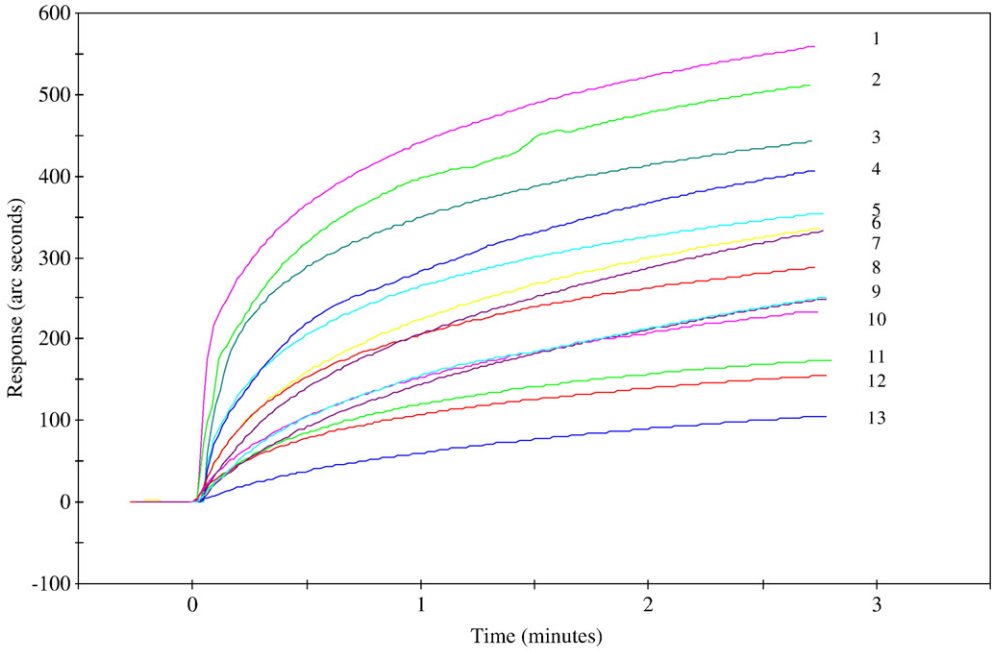


Fig. 1. Immobilization of CpG DNA 107 on a biotin-cuvette. The biotin-cuvette was inserted in the instrument. The data acquisition began with a 5 min baseline data gathering. 20 μl of avidin was later added to the PBST in the cuvette and allowed binding to occur for 10 min. Then washed three times with 50 μl of PBST and collected about 10 min of baseline data, after which 5 μl of biotinylated CpG DNA 107 was added to 45 μl PBST in the cuvette, followed by another collection of binding data about 10 min. Finally washed three times with 50 μl of PBST and collected 5 min of baseline data. *"arc seconds" is the unit of angular measurement applied by biosensor to represent affinity response. One arc second is equal to 1/3600 radian or 0.01592°. In affinity sensor, there will be changes in the light path of laser which reflects from the binding surface of mobilized and immobilized molecules if interaction occurs between the molecules. The change will be greater if higher affinity exists, resulting in the increase of response, the unit of which is arc second. (Response of 100 arc sec represents higher affinity than that of 50 arc sec).

washed rapidly at least seven times with 60 μl PBS/AE and collected about 5 min of data. The next was five times washing with 50 μl 0.1 M HCl followed by seven times washing with 60 μl PBS/AE and 5 min data collection. Then the curve was washed five times with 50 μl 10 mM NaOH, left for 1 min, washed seven times with 60 μl PBS/AE and collected about 5 min of data. The last step was to add BSA solution to a final concentration of 1.5 mg/ml, the cuvette wash five times with 60 μl PBS/AE.



1. Terminalia chebula Retz
2. Radix et Rhizoma Rhei
3. Fructus canarii
4. Fructus Crataegi
5. Spica Prunellae
6. Radix Sanguisorbae
7. Fructus Bruceae
8. Cacumen Platycladi
9. Radix Salviae Mil Tiorrhizae or Radix Gentianae
10. Caulis Sargentodoxae
11. Radix Tripterygii Wilfordii
12. Radix Paeoniae Rubras
13. Radix Arnebiae

Fig. 2. The aqueous extractions from fourteen traditional Chinese herbs had CpG DNA-binding ability. One microlitre of an aqueous extraction from each herb was placed into a cuvette containing 49 μl PBST. After 3 min, the cuvette was washed three times with 50 μl PBST and washed with 0.01 M HCl. Data were performed using FASTplot software.

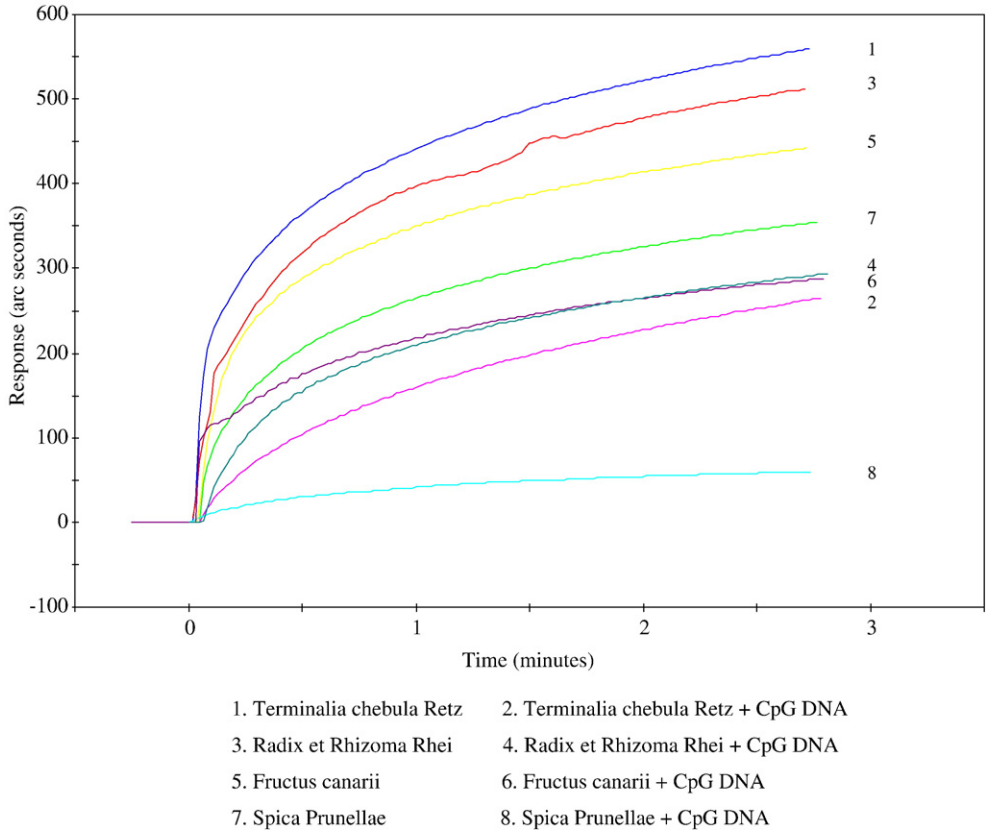


Fig. 3. CpG DNA 107 binding capacity to aqueous extractions. Fifty microlitres each from the aqueous extraction from four traditional Chinese herbs were pre-incubated with 50 μl of 1 OD/ml of CpG DNA 107 for 30 min, and 50 μl of the aqueous extraction was mixed with 50 μl PBST was as control. Two microlitres of the mixture were placed into a cuvette in order to allow binding to CpG DNA 107. Data were performed using FASTplot software.

CpG DNA 107 was immobilized on the biotin-cuvette of biosensor ccording to the manufacturer's instructions (Thermo Labsystem, USA). Briefly, the biotin-cuvette was inserted in the instrument. The data acquisition began with a 5 min baseline data gathering. 20 μl of avidin was later added to the PBST (phosphate buffered saline containing 0.05% Tween 20 (v/v), pH 7.4) in the cuvette and allowed binding to

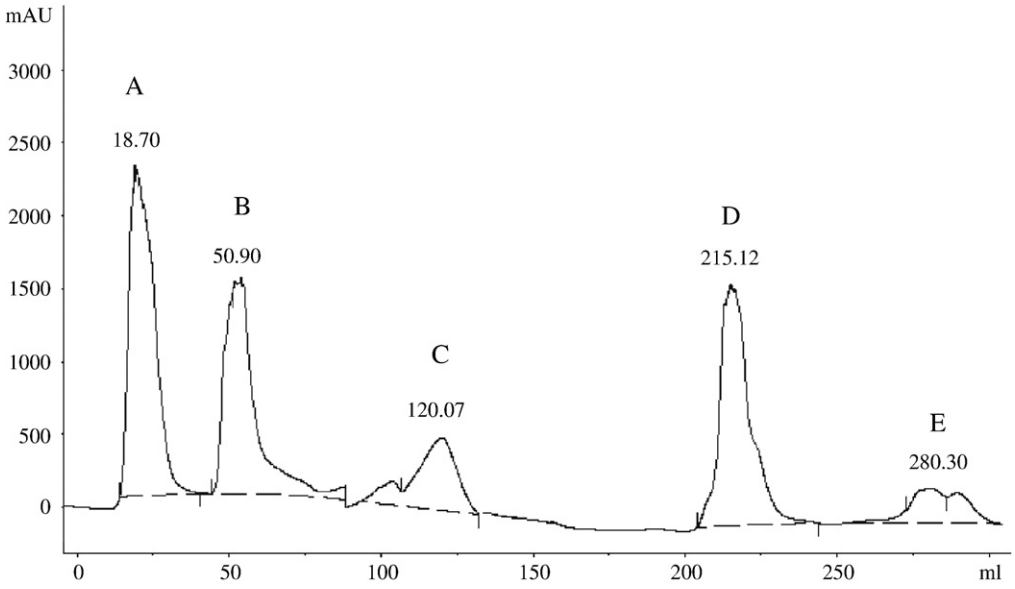


Fig. 4. Isolation of different fractions of *Radix et Rhizoma Rhei* by silica gel column chromatography. The aqueous extractions of *Radix et Rhizoma Rhei* was isolated into five fractions by silica gel column chromatography. One gram of freeze drying supernatants (prepared in 2.2.2) dissolved in 10 ml acetic ether was mixed with 1 g gel and dried at 60 °C. The mixture of the sample and the gel was loaded uniformly onto the upper end of a pre-packed silica gel column (previously prepared as described in Section 2.2.6) and eluted with ethyl acetate, acetone, ethanol and water, respectively. Five fractions were isolated and named A, B, C, D, and E, respectively. Fractions were concentrated and freeze dried for bioactivity evaluations. Each peak in the curve of Fig. 4 represented one fraction. The dotted lines in bottom of the graph represented baseline generated by LC chemostation for the calculation of each peak area.

Table 2
Binding activity of five fractions from HPLC for CpG DNA and lipid A.

	Response (arc seconds)				
	Fraction A	Fraction B	Fraction C	Fraction D	Fraction E
CpG DNA	118.2	8.1	159.5	167.8	79.8
Lipid A	97.8	51.6	145.5	146.1	75.3

Immobilization of CpG DNA 107 or lipid A on the surface of a hydrophobic cuvette or biotin-cuvette. One microlitre of five fractions was added into a cuvette containing 49 μl PBST. After 3 min, the cuvette was washed three times with 50 μl PBST and alternately washed with 0.01 M HCl. Data analyses were performed using the FASTplot software package (Thermo Labsystem, USA).

occur for 10 min. Then washed three times with 50 μl of PBST and collected about 10 min of baseline data. 5 μl of biotinylated CpG DNA was added to 45 μl PBST in the cuvette, followed by another collection of binding data about 10 min. Finally the cuvette washed three times with 50 μl of PBST and collected 5 min of baseline data.

2.2.4. Screening of CpG DNA-binding herbs using affinity biosensor technology

One microlitre of aqueous extractions from each herb was added into a cuvette containing 49 microlitre PBST. After 3 min, the cuvette was washed three times with 50 μl PBST and alternately washed with 0.01 M HCl. Data analyses were performed using the FASTplot software package (Thermo Labsystem, USA).

2.2.5. Capacity of aqueous extractions for binding to CpG DNA

To determine the exact capacity of the aqueous extraction for binding to CpG DNA 107, we selected fourteen aqueous extractions including *Radix et Rhizoma Rhei*, *Radix Gentianae*, *Radix Sanguisorbae* etc., which possessed the higher binding activity to CpG DNA for further study. Fifty microlitres of the aqueous extraction was pre-incubated with 50 μl of 1 OD/ml of CpG DNA for 30 min, with 50 μl of the aqueous extraction mixed with 50 μl PBST as a control. Two microlitres of the mixture was added into the cuvette, and data analyses were performed using the FASTplot software package.

2.2.6. Isolation of fractions with high CpG DNA-binding ability from the water extractions of Radix et Rhizoma Rhei

Freeze dried aqueous extraction from *Radix et Rhizoma Rhei* was obtained as described above in Section 2.2.2, and then was further isolated by silica gel column chromatography. Briefly, 300 g silica gel (for column chromatography use) was deposited in a 500 ml beaker and then baked at 120 °C for 12 h to evaporate water. 1000 ml of acetic ether was added to the dried gel and mixed sufficiently. The wet gel was put into an ultrasonic cleaner (Kunshan ultrasonic instrument Ltd., Kunshan, China) for degassing and then packed into a column. 1 g of freeze dried aqueous extractions dissolved in 10 ml acetic ether was mixed with 1 g silica gel and dried at 60 °C. The mixture of the sample and the gel was loaded uniformly on the upper end of the pre-packed silica gel column and eluted with ethyl acetate, acetone, ethanol and water, respectively. Five fractions was isolated and collected. Each fraction was concentrated by rotary-evaporation (BUCHI Rotavapor R205, Switzerland) and then freeze dried for the assay of its CpG DNA-binding activity. The CpG DNA-binding activity and protection for mice challenged with heat-killed *E. coli* of each fraction was evaluated. And the fraction (Fraction D) with the highest CpG DNA-binding activity was further isolated in order to get monomers in the subsequent procedures.

2.2.7. Bioactive assessment of Fraction D from Radix et Rhizoma Rhei in vitro

2.2.7.1. Affinity assessment for CpG DNA. One microlitre of Fraction D (5, 10, 20, 40, 80 mg/l, respectively) was added into a biotin-cuvette immobilized with CpG DNA 107. After 3 min, the cuvette was washed three times with 50 μl PBST for disassociation and then washed one time with 50 μl 0.01 M HCl for regeneration. Data records were performed using the FASTplot software.

2.2.7.2. Affinity assessment for lipid A. One microlitre of Fraction D (5, 10, 20, 40, 80 mg/l, respectively) was added into a hydrophobic cuvette immobilized with lipid A. After 3 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 records were performed using the FASTplot software.

2.2.7.3. Inhibition TNF-α release induced by LPS or CpG DNA. RAW264.7 (1.5×10^6) were grown in a 48-well plate and incubated for 4 h. Fraction D (20, 40, 80 μg/ml) was added immediately after addition of 100 ng/ml of LPS or 5 μg/ml CpG DNA 1826. After incubation for another 4 h, the supernatants were collected to assess TNF-α level. Herein, PMB (20 μg/ml) was used as positive agent to neutralize LPS.

2.2.7.4. Analysis of TLR9 mRNA expression. 2 ml of the RAW264.7 cells (2.0×10^6 cells/ml) were grown in 6-well polystyrene plates. CpG DNA 1826 (5 μg/ml) was added into the cells. After the addition of CpG DNA 1826 for 4 h, 80 μg/ml of Fraction D was added and incubated with the cells for 2 h. Total RNA was prepared according to the manufacturer's instructions. After DNase I treatment, 2 μg of RNA was reverse transcribed with AMV reverse transcriptase. A master mix containing the reaction buffer, dNTPs, *Taq* polymerase, and 2 μl cDNA in 25 ml reaction mixture was transferred to different PCR tubes. Forward and reverse primers corresponding to different individual genes were added to the PCR tubes and subjected to PCR amplification using primer sets directed against β-actin and TLR9. These reactions were performed for 35 cycles. The annealing temperature was maintained at 51 °C for TLR9; the rest of the conditions included denaturation at 94 °C for 30 s followed by extension at 72 °C for 40 s. The PCR products were determined using 1.0% agarose gel electrophoresis and ethidium bromide staining. Images of the gels were analyzed using the Quantity One software (Bio-Rad, CA, USA), which compares the relative density of objective straps and β-actin. The following primers were used: TLR9 (287-bp product), forward (5′-TGGACGGGAACT GCTACT-3′) and reverse (5′-GCCACATTCTATA CAGGGATT-3′), and β-actin (455-bp product), forward (5′-CCCTGTATGCCTCTGGTC-3′) and reverse (5′-TTTACGGATGTCAACG-3′).

2.2.8. Protection of Fraction D on mice challenged with heat-killed E. coli

Sixty mice were randomly divided into 6 groups (10 mice/group), and then they were injected via the vena caudalis as follows: heat-killed *E. coli* (1.2×10^{10} CFU/kg) in group 1, the Fraction D alone

Table 3
The protection of Fraction C and Fraction D on mice challenged with heat-killed *E. coli*.

Treatment	Numbers of mice	Survivals at day 3	
		Numbers	Survival rate (%)
Heat-killed *E .coli*	10	1	10
Heat-killed *E .coli* + Fraction C (50 mg/kg)	10	1	10
Heat-killed *E. coli* + Fraction C (100 mg/kg)	10	2	20
Heat-killed *E .coli* + Fraction D (50 mg/kg)	10	2	20
Heat-killed *E .coli* + Fraction D (100 mg/kg)	10	6	60*

Survival of mice challenged by heat-killed *E. coli*. Fifty mice were randomly divided into five groups ($n=10$). Groups of mice were treated with: heat-killed *E. coli* (1.2×10^{10} CFU/kg); heat-killed *E. coli* heat-killed *E. coli* (1.2×10^{10} CFU/kg) plus the Fraction C (50 mg/kg); heat-killed *E. coli* (1.2×10^{10} CFU/kg) plus Fraction C (100 mg/kg); heat-killed *E. coli* heat-killed *E. coli* (1.2×10^{10} CFU/kg) plus the Fraction D (50 mg/kg); heat-killed *E. coli* (1.2×10^{10} CFU/kg) plus Fraction D (100 mg/kg). The total injection volume was 0.2 ml per 20 g bodyweight. The general conditions and mortality of the mice were observed for 3 days. *$p<0.05$ compared with heat-killed *E. coli* group.

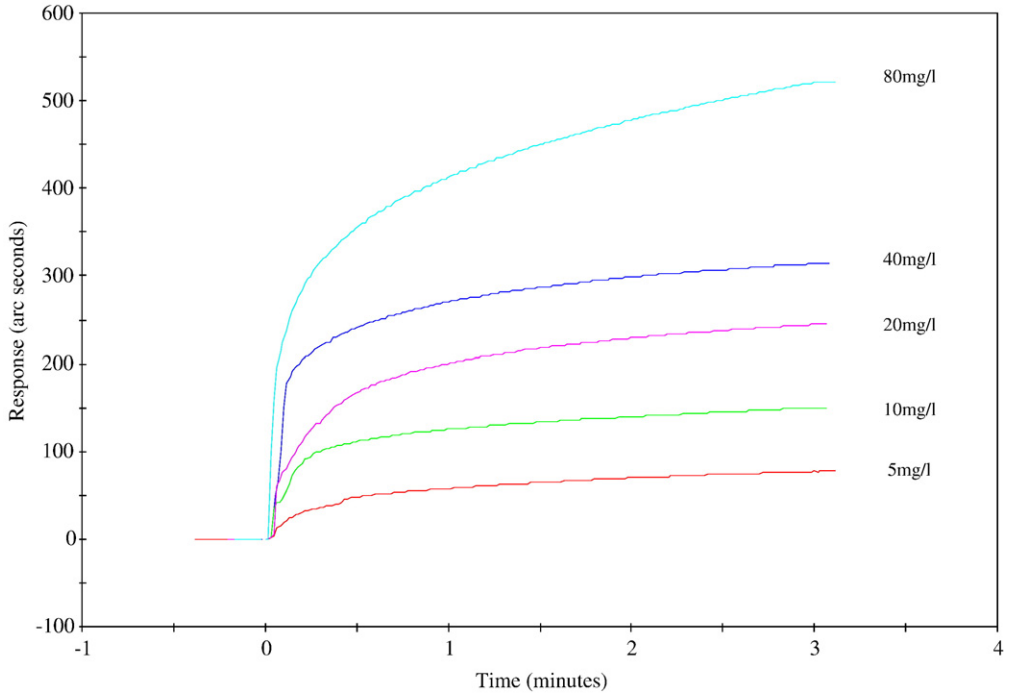


Fig. 5. Binding curve of Fraction D to CpG DNA 107. One microlitre of Fraction D (5, 10, 20, 40, 80 mg/l, respectively) was added into a biotin-cuvette immobilized with CpG DNA 107. After binding for 3 min, the cuvette was washed three times with 50 μl PBST and then washed one time with 0.01 M HCl. Data records were performed using the FASTplot software.

(80 mg/kg) in group 2, heat-killed *E. coli* (1.2×10^{10} CFU/kg) plus the Fraction D (20 mg/kg) in group 3, heat-killed *E. coli* (1.2×10^{10} CFU/kg) plus the Fraction D (40 mg/kg) in group 4, heat-killed *E. coli* (1.2×10^{10} CFU/kg) plus the Fraction D (80 mg/kg) in group 5, and heat-killed *E. coli*.1.2×10^{10} CFU/kg) plus DMX (5 mg/kg) in group 6. The total injection volume was 0.2 ml per 20 g body weight. The general conditions and mortality of the mice were observed for 3 days.

2.2.9. Isolation of ingredients in Fraction D

For analysis, 1 mg/ml of Fraction D was dissolved in methanol and filtered by a 0.22 μm filter membrane. The filtrate was injected into Agilent1200 reverse phase HPLC system. The column was Agilent Eclipse XDB-C18 (4.6×150 mm, 5 μm), and mobile phases was 0.1% acetic acid (phase A) and methanol (phase B). Fraction D was isolated by linear gradient elution with the starting mobile phase (A:B = 95:5) to the ending mobile phase (A:B = 5:95) in 45 min.

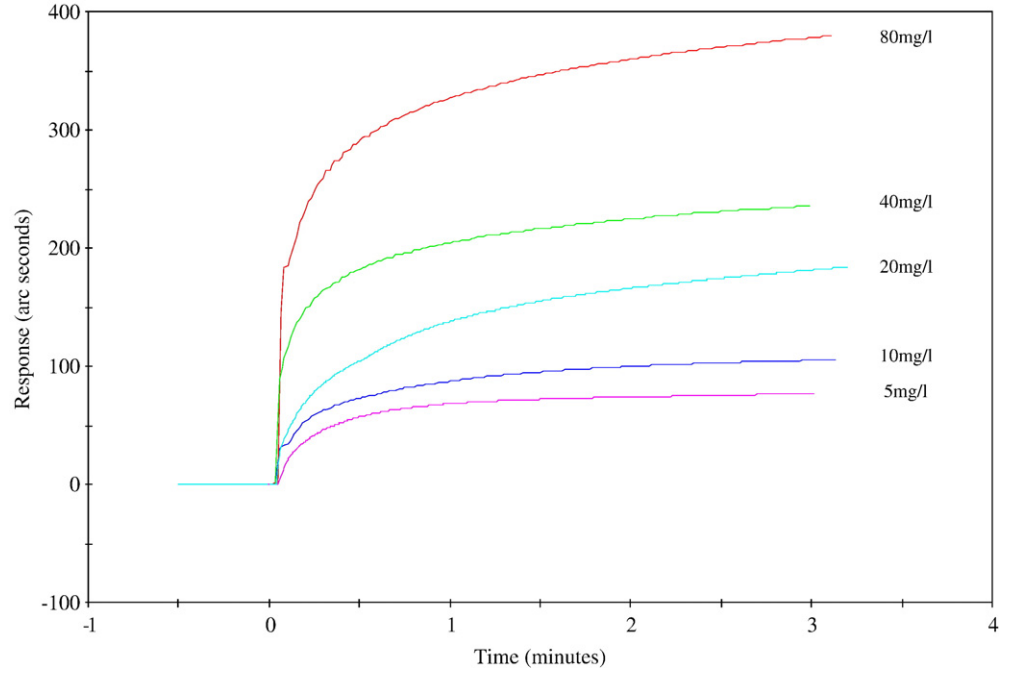


Fig. 6. Binding curve of Fraction D to lipid A. One microlitre of Fraction D (5, 10, 20, 40, 80 mg/l, respectively) was added into a hydrophobic cuvette immobilized with lipid A. After binding for 3 min, the cuvette was washed seven times with 60 μl PBS/AE and alternately washed with 0.1 M HCl, PBS/AE, and 10 mM NaOH, respectively. Data records were performed using the FASTplot software.

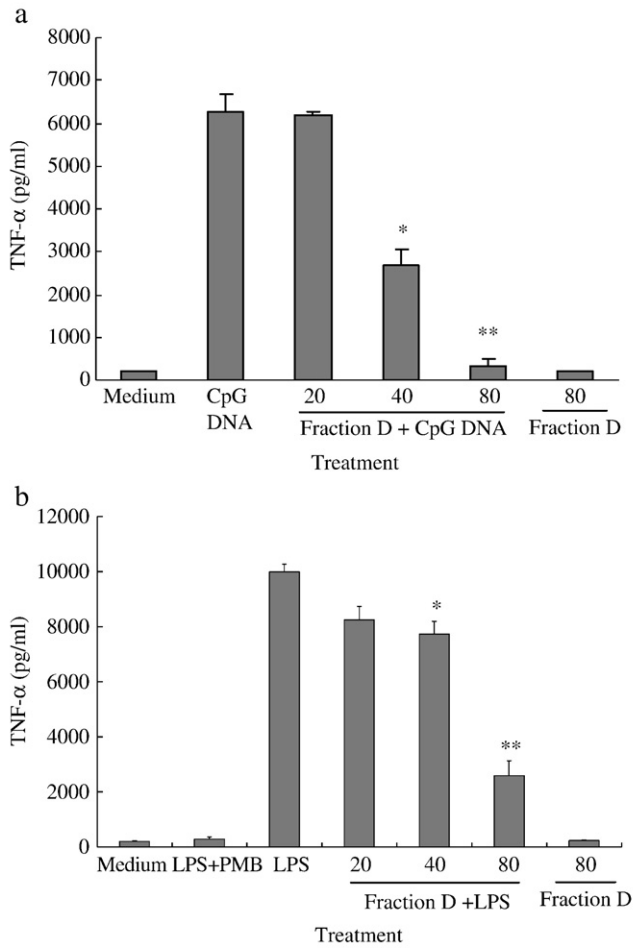


Fig. 7. Inhibition of Fraction D on TNF-α release induced by CpG DNA 1826 (a) and LPS (b). RAW264.7 cells (1.5×10^6) were grown in 48-well plates and incubated for 4 h. The Fraction D (20, 40, 80 μg/ml) and PMB (20 μg/ml, positive control drug) were added, then 5 μg/ml CpG DNA 1826 or 100 ng/ml LPS was added. Incubation for another 4 h, the level of TNF-α in the supernatants was analyzed using TNF-α ELISA kits. *$p<0.05$ compared with LPS or CpG DNA; **$p<0.01$ compared with LPS or CpG DNA.

For preparation, 10 mg/ml of Fraction D was dissolved in 5% methanol and filtered by a 0.22 μm filter membrane. The filtrate was injected into Agilent 1100 reverse phase HPLC system. The column was Agilent ZORBAX SB-C18 PreHT column (21.2×250 mm, 7 μm) and mobile phases was 0.1% acetic acid (phase A) and methanol (phase B). Fraction D was isolated by linear gradient elution with the starting mobile phase (A:B = 95:5) to the ending mobile phase (A:B = 5:95) in 45 min.

2.2.10. Bioactivity assessment of monomers isolated from Fraction D in vitro

2.2.10.1. Affinity assessment for CpG DNA. One microlitre of monomer (2.5, 5, 10 μM) was added into a biotin-cuvette immobilized with CpG DNA 107, respectively. After 3 min, the cuvette was washed three times with 50 μl PBST, and then washed one time with 0.01 M HCl. Data records were performed using the FASTplot software.

2.2.10.2. Inhibition TNF-α release induced by LPS or CpG DNA. RAW264.7 (1.5×10^6) were grown in a 48-well plate and incubated for 4 h. Monomers (20, 40, 80 μg/ml) were added immediately after addition of 100 ng/ml LPS or 5 μg/ml CpG DNA 1826. After incubation for another 4 h, the supernatants were collected to assess TNF-α levels using ELISA kit.

2.2.11. Statistics and presentation of data

All experiments were performed at least three times and representative data were presented. The Chi-squared exact test was used to analyze for the significance of differences in mice mortality among the groups. TNF-α concentrations are expressed as mean ± std. dev. TNF-α data were analyzed with the one-way ANOVA test and post hoc Bonferroni correction was used for multiple comparisons. A p value of less than 0.05 was considered significant, and a value less than 0.01 was considered highly significant.

3. Results

3.1. Immobilization of CpG DNA on biotin-cuvette

CpG DNA 107 was immobilized on the surface of a biotin-cuvette according to the manufacturer's instructions. The image of resonance peak was single and symmetrical, suggesting that the molecules of CpG DNA 107 were uniformly immobilized on the surface of a biotin-cuvette. Calculating the amount of immobilized CpG DNA 107 by subtracting the baseline level from A to B, we obtained the CpG DNA 107 coverage on the sensor surface was 139.5 arc sec (response unit, RU) (Fig. 1).

3.2. Assays for binding to CpG DNA

Among the seventy-eight herbs examined, fourteen herbs were found to possess CpG DNA-binding activities (RU>100 arc sec) (Fig. 2). They were *Radix Salviae Mil Tiorrhizae*, *Radix Arnebiae*, *Spica Prunellae*, *Radix Paeoniae Rubras*, *Radix et Rhizoma Rhei*, *Caulis Sargentodoxae*, *Radix Sanguisorbae*, *Radix Gentianae*, *Fructus Crataegi*, *Radix Tripterygii Wilfordii*, *Fructus Bruceae*, *Terminalia chebula Retz*, *Fructus canarii*, and *Cacumen Platycladi*. However, there were large differences among the herbs (RU ranging from 108.7 to 573.2 arc sec), which suggested there were significant differences of their anti-CpG DNA activities among these herbs.

3.3. Capacity of aqueous extractions for binding to CpG DNA

From the curve of the capacity of aqueous extractions binding to CpG DNA 107, it could be seen that four herbs, which were *Terminalia chebula Retz*, *Radix et Rhizoma Rhei*, *Fructus canarii*, and *Spica*

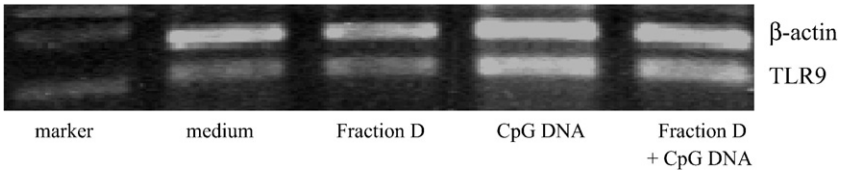


Fig. 8. Effect of Fraction D on TLR9 mRNA expression in RAW264.7 cells. 2 ml of the RAW264.7 cells (2.0×10^6 cells/ml) were grown in 6-well polystyrene plates. CpG DNA 1826 (5 μg/ml) was added into the cells. After the addition of CpG DNA 1826 for 4 h, 80 μg/ml of Fraction D was added and incubated with the cells for 2 h. Total RNA was extracted, and RT–PCR was performed. The signals for TLR9 and β-actin were integrated on a Gel Doc 1000 Mini-Transilluminator (Bio-Rad). The order of the molecular weight markers used was as follows, from top to bottom: 750, 500, and 250 bp.

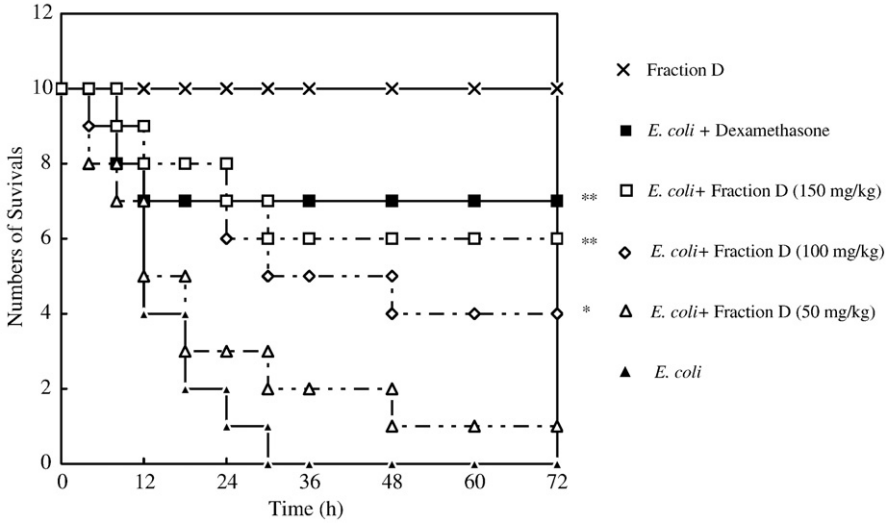


Fig. 9. Survival of mice challenged by heat-killed *E. coli*. Sixty mice were randomly divided into six groups ($n=10$). Mice were injected via the vena caudalis as follows: Fraction D alone (150 mg/kg); heat-killed *E. coli* (1.2×10^{10} CFU/kg) plus dexamethasone (5 mg/kg); heat-killed *E. coli* heat-killed *E. coli* (1.2×10^{10} CFU/kg) plus the Fraction D (150 mg/kg); heat-killed *E. coli* (1.2×10^{10} CFU/kg) plus Fraction D (100 mg/kg); heat-killed *E. coli* (1.2×10^{10} CFU/kg) plus Fraction D (50 mg/kg); heat-killed *E. coli* (1.2×10^{10} CFU/kg) alone. The total injection volume was 0.2 ml per 20 g bodyweight. The general conditions and mortality of the mice were observed for 3 days. *$p<0.05$ compared with heat-killed *E. coli* group; **$p<0.01$ compared with heat-killed *E. coli* group.

Prunellae, had CpG DNA-binding activities after incubation with CpG DNA (Fig. 3). Among these herbs, *Radix et Rhizoma Rhei* was one of the highest. Therefore, it was used for further extraction and purification to get the anti-CpG DNA agent.

3.4. Isolation of active fraction from Radix et Rhizoma Rhei

After the separation with silica gel column chromatography and HPLC, we obtained five fractions (Fig. 4). Fraction D was found to possess high binding activity for CpG DNA 107 (Table 2) and high protection ability for mice challenged with lethal heat-killed *E. coli* (Table 3).

3.5. Bioactivity assessment of Fraction D from Radix et Rhizoma Rhei in vitro

3.5.1. Fraction D possessed high affinities for CpG DNA and lipid A

To test whether Fraction D could possess high affinities for CpG DNA 107 and lipid A, different concentrations of Fraction D were added into the cuvette. The result showed that Fraction D had high affinities not only for CpG DNA but also for lipid A in a dose-dependent manner (Figs. 5, 6).

3.5.2. Fraction D inhibited CpG DNA- and LPS-induced TNF-α release

To confirm whether the protective effect of Fraction D was tightly associated with cytokine reduction, we tested the ability of Fraction D

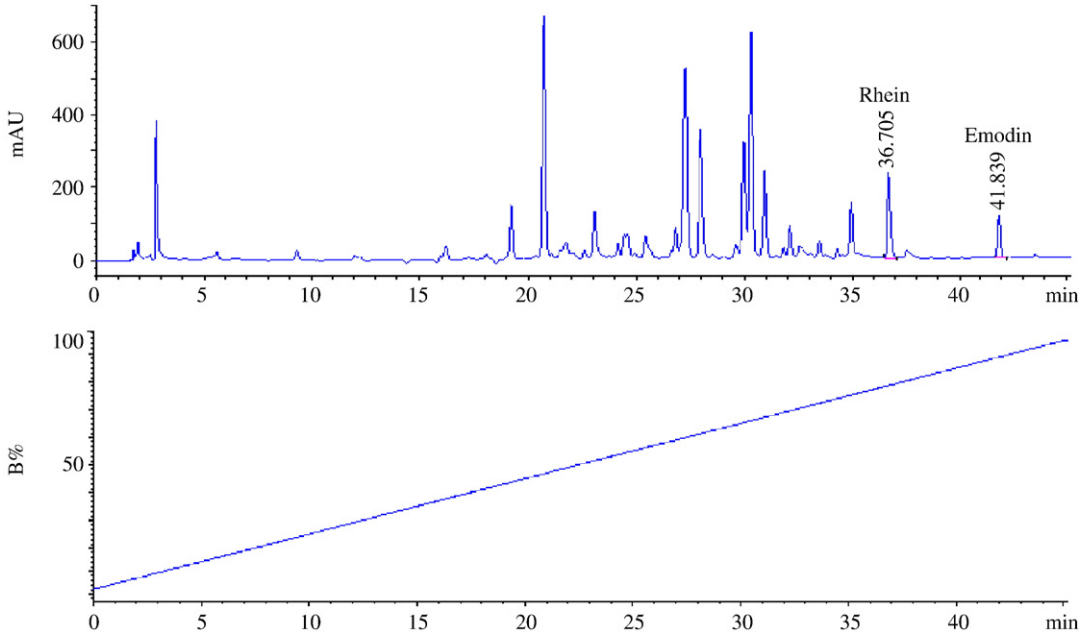


Fig. 10. Analysis of Fraction D by HPLC. Fraction D (1 mg/ml) was dissolved in methanol and filtered. The filtrate was injected onto a C18 column in reserved phase HPLC system (Agilent, USA) eluted with methanol/0.1% acetic acid. The above part showed two monomers, identified later as rhein and emodin, were purified from Fraction D with the retention times at 36.705 min and 41.839 min each. The lower part of this figure showed solvent gradient used for elution. B% represented the proportion of methanol from 5% to 95% in a linear gradient way. The purities of two monomers were more than 90%, as determined by HPLC (data was not shown).

to inhibit TNF-α release from RAW264.7 cells. The result showed that Fraction D (20, 40, 80 μg/ml) inhibited TNF-α release induced by CpG DNA 1826 and LPS in a dose-dependent manner (Fig. 7). Therefore, the concentration of 80 μg/ml was used in the subsequent experiment.

3.5.3. Fraction D reduces TLR9 mRNA expression

For the induction of cytokine release by CpG DNA, TLR signaling is required [12]. In macrophages and monocytes, TLR9 is a pattern recognition receptor for CpG DNA [12]. Accordingly, we investigated the mRNA expression of TLR9 in Fraction D-treated cells. The result showed that CpG DNA 1826 significantly elevated TLR9 mRNA expression in RAW264.7 cells, and Fraction D (80 μg/ml) could effectively decrease the expressions of TLR9 mRNA stimulated by CpG DNA 1826 (Fig. 8).

3.6. Protection of Fraction D on mice challenged with heat-killed E. coli

To test whether Fraction D could protect mice from a lethal challenge with heat-killed *E. coli*, Fraction D was injected into the animals. For mice challenged by *E. coli* (without Fraction D pre-treatment), all died. By contrast, animals pretreated with Fraction D were protected from heat-killed *E. coli* in a dose-dependent manner; Fraction D (100 mg/kg) protected mice to a significant degree ($p<0.05$), and Fraction D (150 mg/kg) had a highly significant protective effect ($p<0.01$) (Fig. 9).

3.7. Isolation of two monomers from Fraction D and identification as rhein and emodin

To get the bioactive ingredient of Fraction D, two monomers with the purities more than 90% were isolated using HPLC method (Fig. 10). The monomers were further identified as rhein and emodin (Fig. 11) after comparing with standard substances by analysis of UV spectrum, thin layer chromatography (TLC) and HPLC (data not shown).

3.8. Bioactive assessment of rhein and emodin in vitro

3.8.1. Rhein and emodin possessed relative high affinities for CpG DNA

To confirm whether rhein and emodin could also possess high affinities for CpG DNA just like Fraction D, the affinities of rhein and emodin for CpG DNA were attested, too. The result showed that both rhein and emodin could bind CpG DNA in a dose-dependent manner (Fig. 12). However, rhein possessed higher binding ability for CpG DNA than emodin did.

3.8.2. Rhein inhibited CpG DNA- and LPS-induced TNF-α release, but emodin only inhibited CpG DNA-induced TNF-α release

To further confirm the bioactivities of rhein and emodin, we observed the abilities of rhein and emodin to inhibit TNF-α release from RAW264.7 cells. The results showed that although rhein could significantly reduce TNF-α release induced by both CpG DNA and LPS, emodin only reduced TNF-α release induced by CpG DNA (Fig. 13). Additionally, rhein in combination with emodin was found to play synergistic inhibitory effects on both CpG DNA- and LPS-induced TNF-α release (Fig. 13). Above results suggested rhein and emodin might

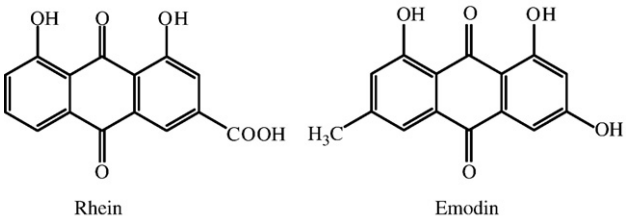


Fig. 11. Structure of rhein and emodin.

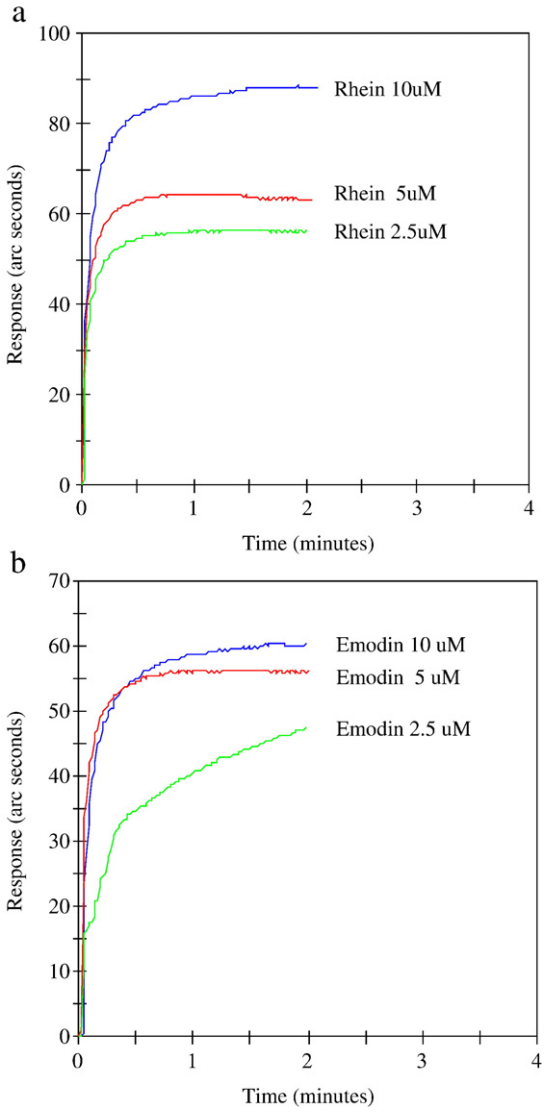


Fig. 12. Binding curve of rhein (a) and emodin (b) to CpG DNA 107. One microlitre of different concentrations of rhein and emodin (2.5, 5, 10 μM) was added into a biotin-cuvette immobilized with CpG DNA 107. After binding for 3 min, the cuvette was washed seven times with 60 μl PBS/AE and alternately washed with 0.1 M HCl, PBS/AE, and 10 mM NaOH, respectively. Data records were performed using the FASTplot software.

be the major contribution to the ability of Fraction D to inhibit CpG DNA-induced TNF-α release.

4. Discussion

To the best of our knowledge, this is the first report showing that both the fraction and the bioactive monomers (rhein and emodin) from traditional Chinese herb, targeting CpG DNA, were able to inhibit proinflammatory cytokine release from RAW264.7 cells induced by CpG DNA. Importantly, we observed Fraction D from *Radix et Rhizoma Rhei* was also capable of protecting sepsis model mice challenged with lethal dosage of heat-killed *E. coli*.

Previously, we successfully established the platform to screen the active ingredients targeting LPS/lipid A from traditional Chinese herbs, and got active monomer [11]. In the present study, we also used affinity biosensor technology to establish the platform to screen the active ingredients targeting CpG DNA from traditional Chinese herbs. Using a CpG DNA-coated surface, the specific interactions of fractions

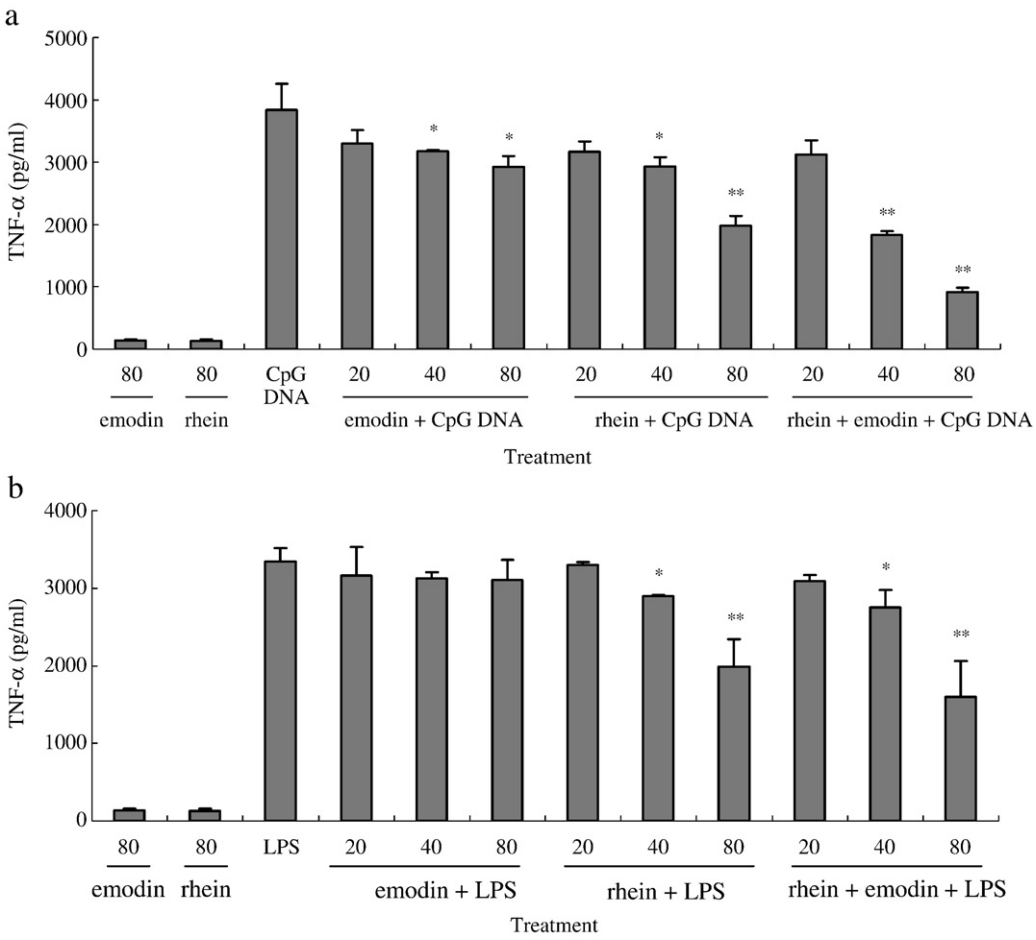


Fig. 13. Inhibition of rhein and emodin on TNF-α release induced by CpG DNA 1826 (a) and LPS (b). RAW264.7 cells (1.5×10^6) were grown in 96-well plates and incubated for 4 h, rhein and emodin (20, 40, 80 μg/ml) were added, and then 100 ng/ml of LPS or 5 μg/ml CpG DNA 107 was added. Incubation was carried out for another 4 h. The level of TNF-α in the supernatants was analyzed using the appropriate ELISA kits. $^{**}p<0.01$ compared with LPS or CpG DNA, $^{*}p<0.05$ compared with CpG DNA, $^{‡}p>0.05$ compared with LPS.

from different traditional Chinese herbs with CpG DNA was investigated. It was confirmed that affinity biosensor technology is a rapid and effective method to screen the active fractions from traditional Chinese herbs. Using this platform, we screened seventy-eight herbs. Among these herbs, fourteen herbs were found to posses CpG DNA-binding activities. And we obtained the active Fraction D from *Radix et Rhizoma Rhei* which could markedly inhibit TNF-α release from RAW264.7 cells induced by CpG DNA or LPS, and protected mice from lethal challenged with heat-killed *E. coli*.

Radix et Rhizoma Rhei is a very important and widely used traditional Chinese herb. Some studies had demonstrated that *Radix et Rhizoma Rhei* possessed pharmacologic effects such as anti-virus, inhibition cancer and treatment of diabetes [13–15]. However, ingredients of *Radix et Rhizoma Rhei* were very complicated. Some of them could play therapeutic effect, but some of them were toxic and even lead to death [16].

Emodin (1, 3, 8-trihydroxy-6-methylanthraquinone) and rhein (4, 5-dihydroxyanthraquinone) are biologically active natural compound that can be chemically classified as anthraquinone derivatives extracted from *Radix et Rhizoma Rhei*. Several studies had showed their effects in the treatment of burn, infection, gallstone, hepatitis, inflammation, and osteomyelitis. Emodin was reported to inhibit LPS-induced NF-κB activation and inflammatory cytokine expression in RAW264.7 macrophages, and synergized with baicalin to suppress TLR4 and IL-6 expressions in pancreas and lung tissues of rats in the setting of acute pancreatitis [17,18]. Rhein was also found to exert anti-inflammatory roles by inhibiting IL-1β-induced activation of MEK/ERK pathway and NF-κB and AP-1 in chondrocytes [19]. In our present study, we obtained an active fraction, Fraction D. Although Fraction D was not a monomer, it had higher binding activity to CpG DNA than the original aqueous extraction. Importantly, although emodin and rhein were the first time to isolated under the direct of targeting CpG DNA, the two monomers were shown to reduce LPS-induced release of TNF-α in accordance with others' findings [17,18].

Interestingly, although we isolated Fraction D under the direct of targeting CpG DNA, Fraction D could bind to not only CpG DNA but also LPS in a dose-dependent manner. Furthermore, Fraction D also inhibited cell activation induced by CpG DNA and LPS. Most significantly, Fraction D protected mice from lethal challenge with heat-killed *E. coli*. As known, heat-killed *E. coli* lacks viability, but CpG DNA and LPS still exist in the cells. Therefore, the sepsis model made by heat-killed *E. coli* can represent the ability of *E. coli* to induce sepsis.

Signaling by TLR family members is required for CpG DNA to induce cytokines release. TLR9 is a pattern recognition receptor (PRR) for CpG DNA, and CpG DNA activates the TLR9-mediated signal transduction pathway to regulate the release of cytokines [12]. Our results demonstrated that Fraction D could inhibit TLR9 mRNA expression in the RAW264.7 cells stimulated by CpG DNA. Therefore, we thought that direct Fraction D-binding to CpG DNA lead to less activation of TLR signal pathway, and less TLR9 mRNA expression.

In our present experiments, rhein and emodin were isolated from Fraction D. Although previous experiments had shown both rhein and emodin significantly inhibited nitrite production from LPS-activated RAW264.7 cells [20], our results here showed that both rhein and emodin reduced CpG DNA-induced TNF-α release, but only rhein

could significantly reduced LPS-induced TNF-α release. Therefore, both rhein and emodin contributed to Fraction D to inhibit CpG DNA-induced TNF-α release, only rhein contributed to Fraction D to inhibit LPS-induced TNF-α release.

In addition, although combination of rhein and emodin, two bioactive monomers from Fraction D, played synergistic inhibitory effect on both LPS- and CpG DNA-induced TNF-α release, the synergistic effect was lower than those of Fraction D. Reasonably, there were probably other active ingredients in Fraction D to play inhibitory effects on both LPS- and CpG DNA-induced TNF-α release although their amount was very small. Therefore, it was significant to further investigate other bioactive ingredients from *Radix et Rhizoma Rhei*.

In conclusion, we have found that *Radix et Rhizoma Rhei* had anti-sepsis ability. Both the Fraction D and the bioactive monomers in Fraction D from *Radix et Rhizoma Rhei* were capable of inhibiting the release of proinflammatory cytokines. Affinity biosensor technology was useful for screening new anti-sepsis agent from traditional Chinese herbs. Fraction D from the *Radix et Rhizoma Rhei* could significantly inhibit LPS- and CpG DNA-induced TNF-α release from RAW264.7 cells by directly binding to LPS and CpG DNA, and down-regulated later TLR9 mRNA expression. Importantly, Fraction D could protect mice from lethal challenge with heat-killed *E. coli*. Rhein and emodin isolated from Fraction D significantly reduced CpG DNA-induced TNF-α release but only rhein reduced LPS-induced TNF-α release. Our results demonstrated that traditional Chinese herbs were the potential candidates to treat sepsis. The effective monomers isolated from traditional Chinese herbs were worth further investigating for drug development.

Acknowledgement

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