www.elsevier.com/locate/jep

The effective components of Huanglian Jiedu Decoction against sepsis evaluated by a lipid A-based affinity biosensor

Gui-Rong Chen, Gang Zhang, Ming-Yu Li, Jing Jing, Jing Wang, Xu Zhang, Brianna Mackie, De-Qiang Dou

PII: S0378-8741(16)30177-5
DOI: http://dx.doi.org/10.1016/j.jep.2016.03.064
Reference: JEP10071

To appear in: *Journal of Ethnopharmacology*

Received date: 13 August 2015
Revised date: 22 March 2016
Accepted date: 30 March 2016

Cite this article as: Gui-Rong Chen, Gang Zhang, Ming-Yu Li, Jing Jing, Jing Wang, Xu Zhang, Brianna Mackie and De-Qiang Dou, The effective components of Huanglian Jiedu Decoction against sepsis evaluated by a lipid A-based affinity biosensor, *Journal of Ethnopharmacology* http://dx.doi.org/10.1016/j.jep.2016.03.064

The effective components of Huanglian Jiedu Decoction against sepsis evaluated by a lipid A-based affinity biosensor

Gui-Rong Chen [a*], Gang Zhang [b], Ming-Yu Li [a], Jing Jing [a], Jing Wang [a], Xu Zhang [a], Brianna Mackie [b], De-Qiang Dou [a*]

[a]. College of pharmacy, Liaoning University of Traditional Chinese Medicine, 77 Life One Road, DD port, Dalian 116600, China (Phone: +86-411-85890147; fax: +86-411-85890288;

[b]. Department of Medicinal Chemistry, Virginia Commonwealth University, USA, 23219

(Received ; final version received)

ABSTRACT

Ethnopharmacological relevance: Huanglian Jiedu Decoction (HJD), the classical recipe for relieving fever and toxicity, has been used for treating sepsis in China for sixteen years. However, the effective components of HJD have not been elucidated until now. Therefore, there is a need to elucidate the effective components of HJD against sepsis on animal models induced by endotoxin (LPS). The affinity force of the effective components of HJD with lipid A was evaluated by a biosensor.

Materials and methods: Lipid A is regarded as the bioactive center of LPS and is always used as a drug target. In order to obtain the effective components of HJD against sepsis, seven fractions from HJD were tested by a biosensor method for assessing the affinity for lipid A. After further separation, the components were isolated from high lipid A-binding fractions and their affinities to lipid A were assessed with the aid of a biosensor. Their activities were then assayed by an *in vivo* experiment administered through a tail vein injection. The levels of LPS, TNF-α, and IL-6 from the blood were found and pathology experiments were performed.

Results: Three out of the seven fractions exhibited high lipid A-binding affinities. Berberine, baicalin and geniposide were obtained from the three high lipid A-binding fractions. The animal experiments indicated that the levels of LPS, TNF-α and IL-6 in the medicated treatment groups were much lower than that of the model group (***P*<0.01). The medicated treatment groups exhibited stronger protective activities on varying organs in the animal model.

Conclusions: Berberine, baicalin and geniposide could neutralize LPS by binding with lipid A and then reduce the release of IL-6 and TNF-α induced by LPS. Furthermore, berberine, baicalin and geniposide exhibited protective activities on varying organs compared to the animal model established by the LPS-induced. These results validate that the components from HJD neutralized LPS and then depressed the release of IL-6 and TNF-α induced by LPS. This gives further evidence that HJD would be a suitable treatment for sepsis and protecting vital organs.

Keywords
Huanglian Jiedu Decoction, lipid A, sepsis, the effective components, biosensor method

1 Introduction

In most Gram-negative bacteria, lipopolysaccharide (LPS) is indispensable for bacteria survival. Once toxic LPS is released, it plays a key role in Gram-negative infections in humans (Molinaro et al., 2015). LPS is composed of three distinct domains: lipid A, a core oligosaccharide and an O-antigen (Gattis et al, 2013). Lipid A is necessary to activate Toll-like receptor 4 (TLR4) signaling pathway by combining with TLR4 co-receptor protein myeloid differentiation factor 2 (MD-2) (Park et al., 2009). The TLR4/MD-2 complex receptor is bound by lipid A and results in two different signaling transduction pathways and leads to the release of pro-inflammatory-cytokines (Zhang and Ghosh, 2001; Ohto et al., 2007), which consequently leads to a significant enhancement of the host to resist infection. However, a large amount of LPS may cause an uncontrolled and massive immune response caused by circulation which can lead to more severe symptoms of sepsis. Research shows that LPS is the main promoter for mediating sepsis (Gao and Tsan, 2003; Smith et al., 2003; Takeuchi et al., 2001; Diebold et al., 2004); therefore, targeting LPS has drawn attention from the field of critical care and emergency medicine research.

Inflammatory response begins when a negatively charged phosphoric acid of the disaccharide structure in lipid A binds to a positively charged amino acid residue of

the cell-membrane (Yu et al., 2003). Polymyxin B (PMB), a well-established active compound, plays a part in neutralizing LPS by combining with the negative ion of lipid A. Therefore, lipid A was coated on the surface of the sample pool of a biological sensor and then the affinity was detected with different concentrations of the mobile phase PMB. The affinity value, *Kd*, was reported in the literature as *Kd*=10-100 nM, and was calculated by matching the FASTfit software (Ried et al., 1996). The biosensor system has shown advantages of convenience, speed, and reliability in screening against the lipid A ligand.

HJD is a traditional formula consisting of four herbs: *Coptis chinensis Franch.*, *Scutellaria baicalensis Georgi*, *Phellodendron amurense Rupr.* and *Gardenia jasminoides J.Ellis.* HJD is used for the treatment of clinical disease such as sepsis, organ dysfunction syndrome, hypertension, cerebrovascular disease, Alzheimer's disease, pneumonia, meningitis and encephalitis (Wang and Xu, 2000; Fang et al., 2001; Ma et al., 2000; Wang et al., 2007; Liu et al., 2007; Li et al., 2006; Dai and Gao, 2000). Animal and clinical experiments demonstrated that treatment with HJD exhibits strong activity in the sepsis model, including sepsis patients from an intensive care unit (Xiao and Huang, 2009; Huang et al., 2012), and rats induced with cecal ligation and puncture (CLP) (Wei et al., 2013). However, the effective components of HJD have not yet been elucidated. Therefore, HJD was proposed to combine with lipid A, the bioactive center of LPS, for treating sepsis in this experiment. Lipid A was used as the target for screening higher affinity fractions of HJD through a biosensor method. After further separation, the effective components were isolated from the higher affinity fractions. Their affinities were assessed by a biosensor and activity assessments were carried out *in vivo*.

2 Materials and methods

2.1 Reagents

The following reagents were acquired: LPS (Sigma-Aldrich, Shanghai), Lipid A (Sigma-Aldrich, Shanghai), Polymyxin B (Sigma-Aldrich, Shanghai)), the rat TNF-α and IL-6 ELISA kit (liquid, enzymatic method, Nanjing Jiancheng Bioengineering

Institute, Nanjing), Quantitative chromogenic tachypleus amebocyt lysati (Chinese Horseshoe Crab Reagent Manufactory Co., Ltd., Xiamen). Silica gel (Qingdao Marine Chemical Factory, QingDao), Macroporous resin AB-8 (Cangzhou Sage Chemical Co. Ltd., Hebei).

2.2 Chemicals

The four traditional Chinese herbs, *Coptis chinensis Franch., Scutellaria baicalensis Georgi*, *Phellodendron amurense Rupr.* and *Gardenia jasminoides J.Ellis* were all commercially available as dry matter and were purchased from Liaoning China Pharmacy Co., Ltd. (Table 1) and identified by professor Yan-Jun Zai (College of pharmacy, Liaoning University of Traditional Chinese Medicine, China). Voucher specimens were deposited in the herbarium of Liaoning University of Traditional Chinese Medicine for drug control. The dosages of herbs in HJD are based on the theory of Chinese Materia Medica. These herbs were processed into decoction by the Pharmacy Department of General Hospital of Liaoning University of Traditional Chinese Medicine (300 mL per bottle, batch no. 1302102). The quality of HJD was assessed by high liquid chromatography (HLPC) (Chen et al., 2012). Using HPLC, the contents of berberine, baicalin and geniposide in HJD were determined to be 4.6 mg/g, 7.8 mg/g and 12.4mg/g, respectively. Berberine, baicalin and geniposide were prepared from HJD by College of Pharmacy of Liaoning University of Traditional Chinese Medicine (batch no.1307X01, 1310H03 and 1401Z07).

Table 1 Information regarding the herbs

Herbs	Origin (China)	Specimen no.	Dosage (g)
Coptis chinensis Franch.	Sichuan	HL-08-32	550
Scutellaria baicalensis Georgi	Neimeng	HQ-12-16	500
Phellodendron amurense Rupr.	Liaoning	HB-02-24	500
Gardenia jasminoides J.Ellis	Jiangxi	ZZ-23-05	550

2.3 Equipment

An affinity biosensor (Thermo, USA), a light microscope (BX51, Olympus, Japan), a freezing microtome (Thermo, USA), and a Hitachi-H7170 automatic biochemical

analyzer (Hitachi, Japan).

2.4 Animals

Balb/c mice (4-6 weeks old) were obtained from the Experimental Animal Center of Liaoning Benxi Changsheng Biotechnology Co. Ltd. (Liaoning, China). An equal number of male and female mice were used. The weight of the mice on the day of the experiments were 20±2 g. All of the studies were guided by the Animal Center of Liaoning University of Traditional Chinese Medicine, and the animal performance protocol was approved by the ethics committee of the institution.

2.5 Preparation of the fractions of HJD

990 g of the mixed Chinese medicine (*Coptis chinensis Franch., Scutellaria baicalensis Georgi, Phellodendron amurense Rupr.* and *Gardenia jasminoides J.Ellis* = 9:6:6:9) was measured in proportion. Water was used as the solvent to extract the components twice for 1 h each. The extract (fraction 1) was obtained with the treatment of hot filtration. When the hot extract cooled to room temperature, precipitation and supernatant were formed. The precipitation (fraction 2) and supernatant (fraction 3) were obtained with the treatment of centrifugal separation. The supernatant (fraction 3) was concentrated under vacuum to 0.25 g/mL and then underwent separation through a macroporous resin column with distilled water and fifty percent ethanol as the mobile phase. Fractions 4 and 7 were obtained by collecting the eluent separately. Fraction 2 was purified by polyamide column chromatography with distilled water and fifty percent ethanol as the mobile phase. Subsequently, fractions 5 and 6 were obtained by treating the elution in the same way mentioned above.

2.6 Affinity assessment of the fractions form HJD

The affinity assessment was performed according to reference (Jiang et al., 2004; Genfa et al., 2005). In detail, lipid A was immobilized on the surface of a hydrophobic cuvette (Thermo Labsystem, USA) according to the manufacturer's instructions. In addition, the hydrophobic cuvette was pretreated by 2-propanol and PBS/AE (containing 0.025% (w/v) sodium azide and 1 mM EDTA, pH 7.4). 10 μL of 2-propanol and 10 μL of lipid A (20 mg/mL in chloroform) were mixed in the cuvette

and allowed to bind for 1 min. After the cuvette was washed seven times with 60 μL PBS/AE, the data was collected. Next, the cuvette was alternately washed with 0.1 M HCl, PBS/AE and 10 mM NaOH seven times and the data was collected after the seventh PBS/AE wash. The sensor surface was covered with lipid A for 251 arc sec. Different concentrations of PMB were added after lipid A was immobilized on the surface of the hydrophobic cuvette, and then a binding curve was generated. The K_d values were assayed with affinity Sensors IAsys. Additional analyses were carried out by the FASTplot and FASTfit softwarepackages (Thermo Labsystem,USA). We designed an experiment using the Affinity Sensors IAsys Plus system in order to determine the aforementioned components for binding to lipid A. The fractions were prepared from HJD and used to screen for high binding activity with lipid A. The details of the experiment were carried out as mentioned above. 5 μL of the components was added into the cuvette, and data was collected and analyzed using FASTplot software.

2.7 Compounds were prepared from the higher affinity fractions

Fractions 5-7 from HJD were found to have higher binding affinity for lipid A. Fractions 5 and 7 were treated with silica gel column chromatography and dichloromethane-methanol was used as the eluent for further separation and purification. Compound 1 was obtained with the treatment of 95:5 dichloromethane-methanol eluent of fraction 5, followed by concentration and recrystallization of the fraction in ethanol. Compound 2 was obtained by treating fraction 7 with a 90:10 dichloromethane-methanol eluent, followed by recrystallization in methanol. Fraction 6 was purified by silica gel column chromatography and eluted with 25:1, 10:1, 5:1 and 2:1 dichloromethane-methanol. Compound 3 was obtained with the treatment of fraction 6 with 10:1 dichloromethane-methanol and purified by silica gel column chromatography and recrystallization in methanol. Compounds 1-3 were purified and their structures were characterized by ^{1}H NMR and ^{13}C NMR spectra.

2.8 Affinity assessment of the compounds form HJD

Affinities of the compounds were measured as described previously. The *Kd* values

of the compounds with the lipid A monomer was measured as described previously. A binding curve was generated, and *Kd* values were found using the FASTplot and FASTfit software packages (Thermo Labsystem, USA).

2.9 The in vivo pharmacological evaluation for compounds 1-3

The test animals were housed at room temperature, humidity 55-60%. The equivalent dose for each compound was determined by quantitative determination of HJD by high performance liquid chromatography. On the basis of equivalent dose of HJD, berberine (10 mg/Kg) and baicalin (17 mg/Kg) and geniposide (21 mg/Kg) were offered by tail vein injection for 2 consecutive days and no death of mice occurred in this experiment. These results indicate that compounds 1-3 were safe and nontoxic in dosage. Balb/c mice (4-6 weeks weighing 20±2 g) were randomly divided into 5 groups (50 mice/group) and were intravenously injected as follows: LPS (18 mg/Kg) in group 1, LPS (18 mg/Kg) plus berberine (10 mg/Kg) in group 2, LPS (18 mg/Kg) plus baicalin (17 mg/Kg) in group 3, LPS (18 mg/Kg) plus geniposide (21 mg/Kg) in group 4. Group 5 was the control group and was treated with an injection of saline. The total injection volume was 0.2 mL per 20 g bodyweight. The general conditions and mortality of the mice were observed for 2 days.

2.10 Measurement of LPS and IL-6 and TNF-α in blood

Blood samples (0.5 mL) were taken at 2, 6, 12, 24 and 48 h after inception of the experiment, and the serum was stored at -20℃ for subsequent LPS and TNF-α and IL-6 assays. Additionally, 100 μL of the plasma from groups 1-5 were diluted in 200 μL of anticoagulants, and then the solutions were incubated in 70℃ water for 10 min in order to inactivate heparin. The LPS level in the solutions were assayed with the treatment of quantitative chromogenic tachypleus amebocyt lysati test.

2.11 Pathological investigation of the tissues

The heart, liver, lung and kidney of mice were removed for pathological investigation following the treatment of formalin-fixed, paraffin embedded and sliced and stained tissues with hematoxylin-eosin (H&E). Slides were visualized by standard light microscopy.

2.12 Statistical analysis

Statistical analysis was carried out by using SPSS 17.0 software. The data is expressed as mean ± SD. The data was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett method and Chi-squared test. A *p* value of less than 0.05 was considered significant, and a value less than 0.01 was considered highly significant.

3. Result

3.1 The affinity assay of the fractions from HJD for lipid A

To define the specificity of PMB in binding to lipid A, the K_d value was measured by FASTfit. The affinity of the components with lipid A were compared with that of PMB, a high affinity binder of lipid A. When PMB was pre-incubated with lipid A, lipid A was immobilized on the surface of a hydrophobic cuvette as described in the materials and methods. Different concentrations of PMB were added to the immobilized surface, and the K_d value was measured. The K_d value of PMB was 11.1nM. The affinity assays of fractions for lipid A were carried out as described in the methods (Fig. 1-2).

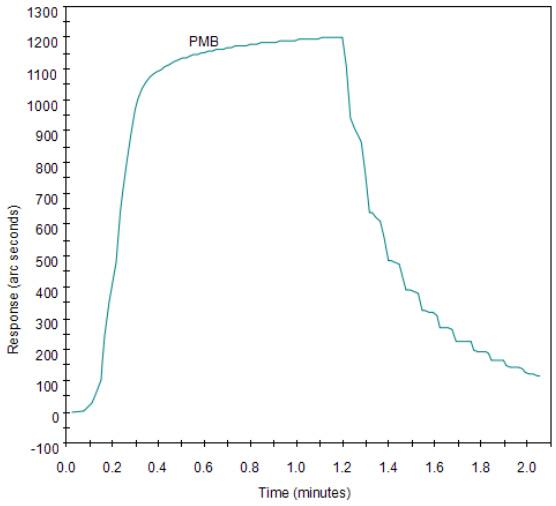
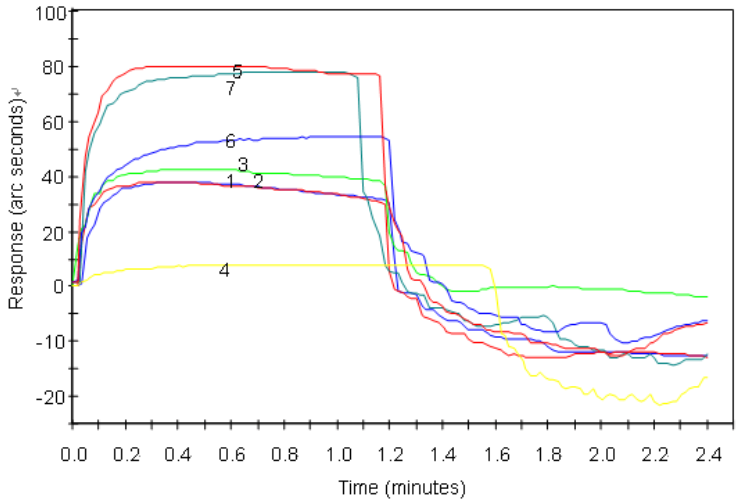


Fig. 1 Affinity of the PMB with lipid A.



1 HJD 2 precipitation 3 supernatant 4 the supernatant eluent of distilled water 5 the precipitation eluent of distilled water 6 the precipitation eluent of fifty percent ethanol 7 the supernatant eluent of fifty percent ethanol

Fig. 2 Affinities of the fractions with lipid A.

3.2 Structure elucidation for compounds 1-3.

Compound 1 was obtained as a yellow needle crystal. Two methoxyls were observed by the signals at δ 4.10 (3 H, s) and δ 4.20 (3 H, s) in the ^{1}H NMR spectrum and their corresponding carbon signals were attributed at δ 57.2 and δ 57.7, respectively. The signals at δ 3.25 (2 H, t) and δ 4.92 (2 H, t) in the ^{1}H NMR spectrum and the signals at δ 62.6 and δ 28.2 in the ^{13}C NMR spectrum indicate the presence of a -CH_2CH_2-N- fragment. The proton signal at δ 6.10 (2 H, s) in the ^{1}H NMR spectrum and the carbon signal at δ 103.7 in the ^{13}C NMR spectrum belonged to methylene-dioxy group. Two aromatic protons appeared at δ 7.98 (2 H, d) and δ 8.09 (2 H, d) in the ^{1}H NMR spectrum, indicating one 1,2,3,4-tetrasubstituted phenyl group. Two aromatic protons emerged at δ 6.95 (H, s) and δ 7.64 (H, s) in the ^{1}H NMR spectrum, suggesting one 1,2,4,5-tetrasubstituted phenyl group. By comparing the spectral data with those of berberine mentioned in the literature (Qin and Wang, 2003), the complete molecule of compound 1 was established with the help of ^{1}H NMR and ^{13}C NMR experiments. The structure of 1 was determined to be 7, 8, 13, 13α-tetradehydro-9, 10-dimethoxy-2, 3-(methylenedioxy) berbinium.

^{1}H NMR (CD_3OD): δ 3.25 (2 H, t, *J*=6.6 Hz), δ 4.92(2 H, t, *J*=6.35 Hz), δ 4.10 (3 H,

s), δ 4.20 (3 H, s), δ 6.10 (2 H, s), δ 6.95 (H, s), δ 7.64 (H, s), δ 7.98 (H, d, *J*=9.05 Hz), δ 8.10 (H, d, *J*=9.1 Hz), δ 8.68 (H, s), δ 9.76 (H, s).

^{13}C NMR (CD_3OD): δ 106.5, δ 152.0, δ 149.9, δ 109.4, δ 123.3, δ 139.7, δ 128.2, δ 135.2, δ 124.5, δ 121.9, δ 145.8, δ 152.2, δ 121.5, δ 146.4, δ 62.6, δ 28.2, δ 131.9, δ 57.7, δ 57.2, δ 103.7.

Compound 2 was obtained as a yellow powder. Compound 2 was reacted with hydrochloric acid-magnesium and further qualitative identification was carried out by a Molisch reaction, suggesting that compound 2 belonged to the flavonoid family. A single substituted phenyl was observed by the proton signals at δ 7.49 (3 H, m) and δ 7.93 (2 H, m). The carbon signals at δ 184.4 and δ 172.1 belonged to the carboxyl group of keto and carboxylic acid. The signal appearing at δ 6.72 (H, s) indicated that the ortho-position was glycosidated. The proton signals appearing at δ 4.16 (H, d, J=9.75 Hz), δ 5.16 (H, d, J=7.55 Hz) and δ 3.33-δ 3.45 (3 H, m) in the ^{1}H NMR spectrum and the six carbon signals emerged at δ 102.3, δ 76.9, δ 74.4, δ 76.7, δ 72.9 and δ 172.0 in the ^{13}C NMR spectrum belonged to glycosyl group. By comparing the spectral data with those of baicalin mentioned by the literature (Baker and Bishop, 2004), the structure of 2 was established to be 7-D-glucopyranuronosyloxy)-5,6-dihydroxy-2-phenyl -4H-1-benzopyran-4-one.

^{1}H NMR (CD_3OD): δ 3.40 (3 H, m), δ 4.16 (H, d, *J*=9.8 Hz), δ 5.16 (H, d, J=7.55 Hz), δ 6.72 (H, s), δ 6.95 (H, s), δ 7.49 (3 H, m), δ 7.93 (2 H, m).

^{13}C NMR (CD_3OD): δ 166.2, δ 107.9, δ 184.5, δ 151.4, δ 132.1, δ 152.6, δ 95.8, δ 148.1, δ 105.6, δ 132.5, δ 127.5, δ 130.2, δ 133.1, δ 130.2, δ 127.5, δ 102.3, δ 74.4, δ 76.7, δ 72.9, δ 76.9, δ 172.1.

Compound 3 was obtained as a white powder. One methoxyl group was observed by the signal at δ 3.71 (3 H, s) in the ^{1}H NMR spectrum and the signal at δ 51.7 in the ^{13}C NMR spectrum of compound 3. The carbon signal appearing at δ 169.5 was deduced to be an ester carbonyl group. The ^{13}C NMR signals at δ 153.3, δ 112.6, δ 144.8 and δ 128.4 and the proton signals at δ 5.79 (1 H, s) and δ 7.51 (1 H, s), indicated the presence of two -CH=C- fragments. The proton signals appearing at δ 4.70 (H, d, J=7.9 Hz), δ 5.16 (H, d, J=7.55 Hz) and δ 3.18-δ 3.45 (3 H, m) in the ^{1}H

NMR spectrum and the six carbon signals emerging at δ 100.4, δ 74.9, δ 78.4, δ 71.5, δ 77.9 and δ 62.7 in the ^{13}C NMR spectrum, indicated there is an additional glycosyl group. By comparing the spectral data with those of geniposide mentioned by the literature (El-Naggar et al., 1980), the structure of 3 was determined to be 1-D-glucopyranosyloxy-1,4a,7,7a-tetrahydro-7-hydroxy-7-(hydroxymethyl)-methyl ester.

^{1}H NMR (CD_3OD): δ 2.71 (H, t, *J*=7.6 Hz), δ 2.80 (H, dd, *J*=8.3,16.2 Hz), δ 3.16 (H, m), δ 3.31 (3 H, m), δ 3.71 (3 H, s), δ 4.17 (H, d, *J*=14.5 Hz), δ 4.29 (H, d, *J*=14.4 Hz), δ 4.70 (H, d, *J*=7.9 Hz), δ 5.16 (H, d, *J*=7.6 Hz), δ 5.79 (H, s), δ 7.51 (H, s).

^{13}C NMR (CD_3OD): δ 98.3, δ 153.3, δ 112.6, δ 39.7, δ 36.6, δ 128.4, δ 144.8, δ 47.1, δ 61.4, δ 169.5, δ 51.7, δ 100.4, δ 74.9, δ 78.4, δ 71.5, δ 77.9, δ 62.7.

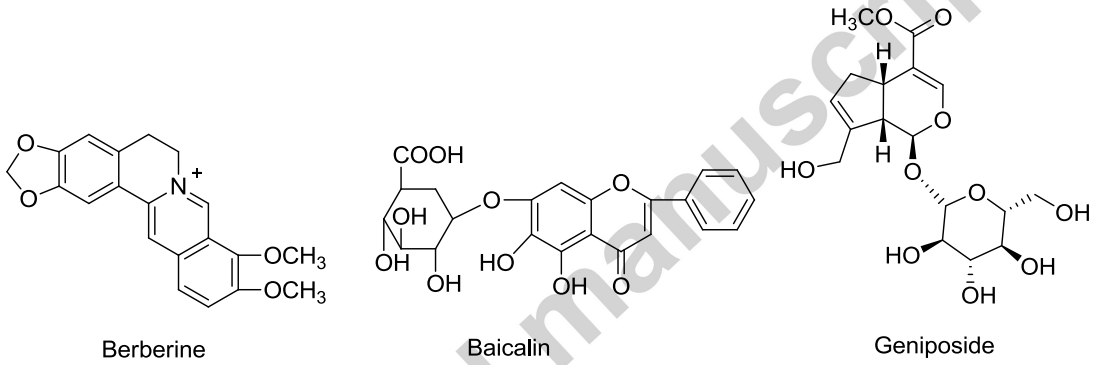
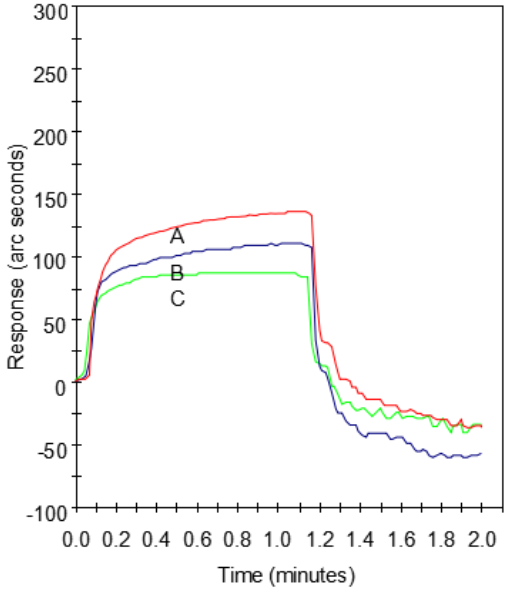


Fig. 3 Structures of the three compounds

3.3 The affinity assay of the compounds from HJD for lipid A

The affinity assays of the compounds with lipid A were carried out as described in the methods (Figure 4). The K_d values of the compounds berberine, baicalin and geniposide were 40.8, 38.3 and 41.5 nM, respectively.



A berberine B baicalin C geniposide

Fig. 4 Affinities of the compounds with lipid A.

3.4 The activity assessment of compounds 1-3

3.4.1 Protecting mice from LPS challenge

In order to test the activity of protective organs in mice from lethal challenges with LPS, the test animals were injected with berberine, baicalin and geniposide after lethal challenges with LPS (18 mg/kg). In this model, among 20 mice were challenged by LPS and only 6 survived. Compared to this model, animals treated with berberine, baicalin and geniposide were protected from LPS induced lethality. The results are shown in Table 2.

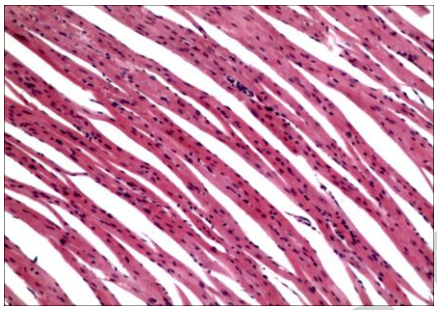
Table 2 The protective activity of compounds 1-3 from LPS challenge.

Group	n	Dose (mg/kg)	LPS (mg/kg)	Mortality	Survival rate (%)
Model	20	0	18	14	30
Berberine	20	10	18	11	45*
Baicalin	20	17	18	10	50**
Geniposide	20	21	18	10	50**

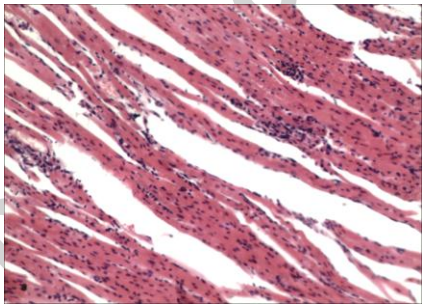
*$p < 0.05$ compared with model group; **$p < 0.01$ compared with model group. The Chi-squared test was used to analyze the data.

3.4.2 Pathological investigation

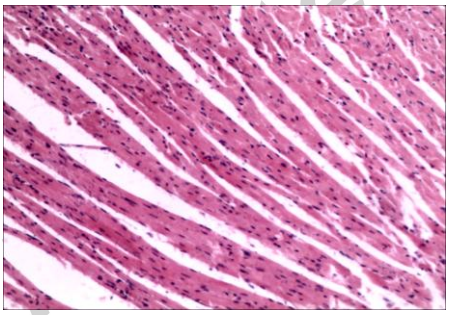
Histological analysis of these organs show that multiple organ injury appeared in the model animals. The results of H&E staining revealed that the model group exhibited significant inflammatory cell infiltration, cell disorder, swelling, congestion, incrassation, morphological and structural changes in the organs. The medicated treatment groups exhibited light inflammatory cell infiltration, regular arrangement in cells, mild edema and relieved congestion and normal morphology and structure. Berberine displayed strong protective activity on the heart, liver and kidney, baicalin exhibited strong protective activity on the heart and liver, and geniposide displayed strong protective activity on the heart, liver and lung. The results are shown in Fig. 5. All of the medicated treatment groups exhibited protective activity on varying organs despite LPS induced challenges as compared to the model group.



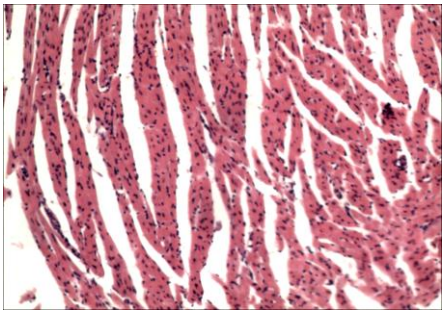
Control group



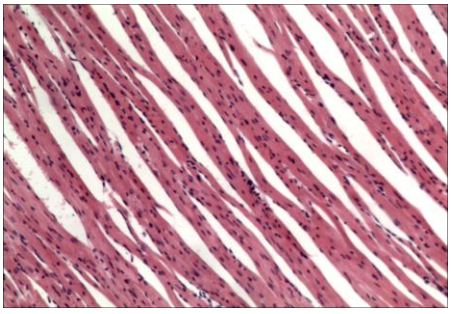
Model group



Berberine group

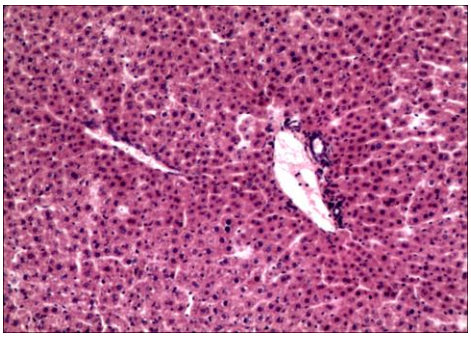


Baicalin group

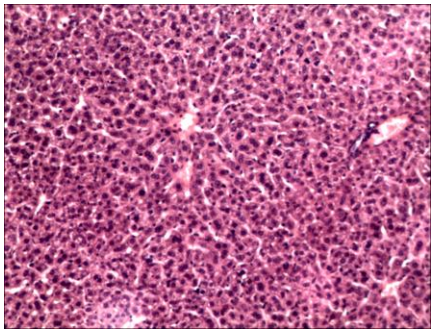


Geniposide group

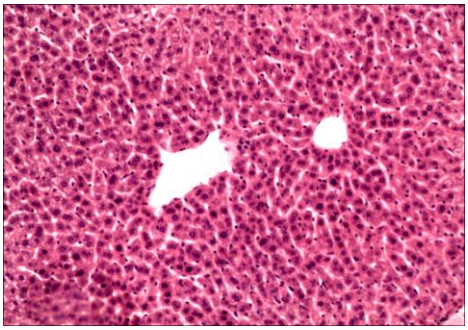
(a)



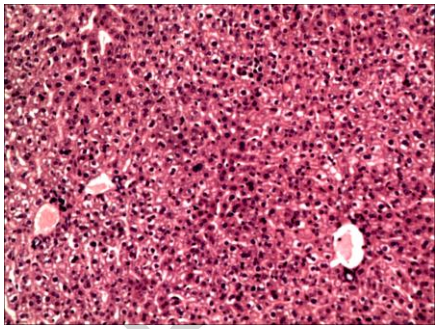
Control group



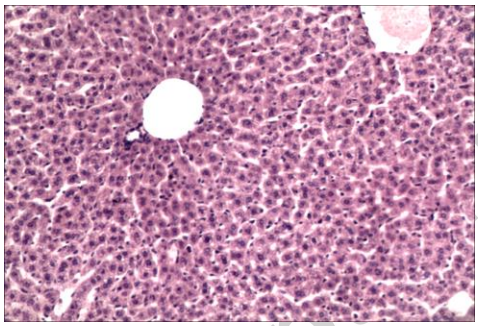
Model group



Berberine group

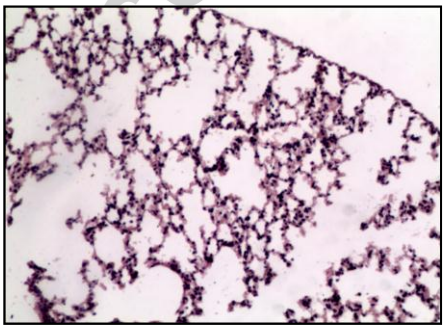


Baicalin group

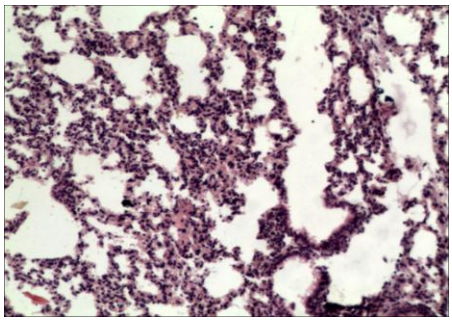


Geniposide group

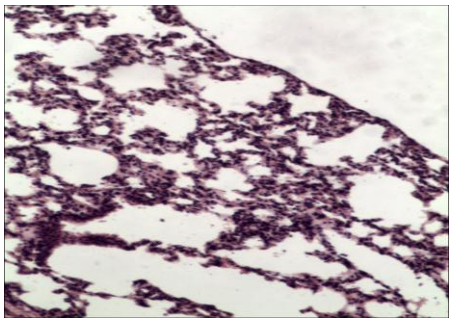
(b)



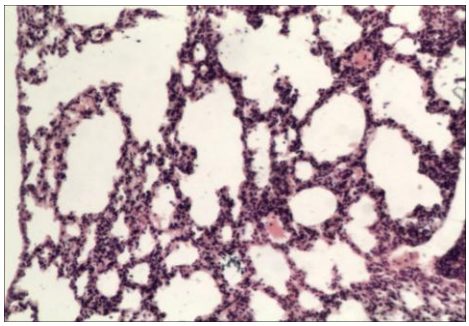
Control group



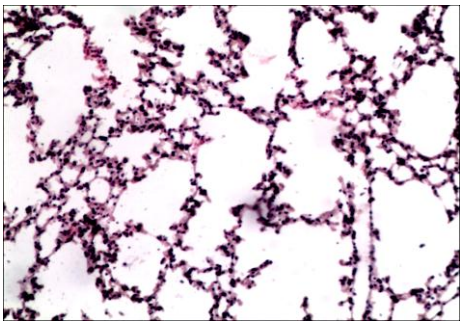
Model group



Berberine group

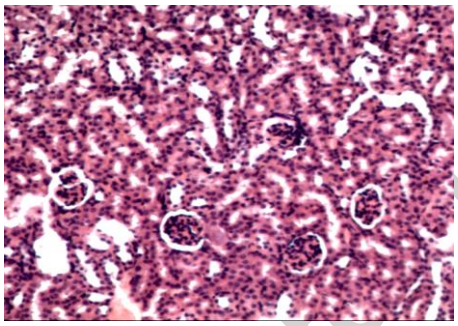


Baicalin group

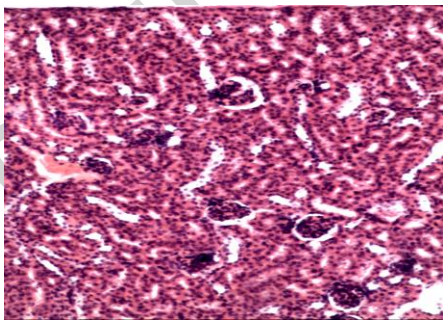


Geniposide group

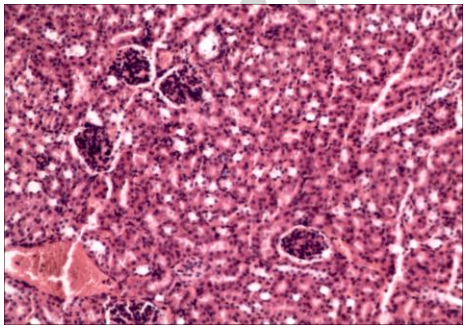
(c)



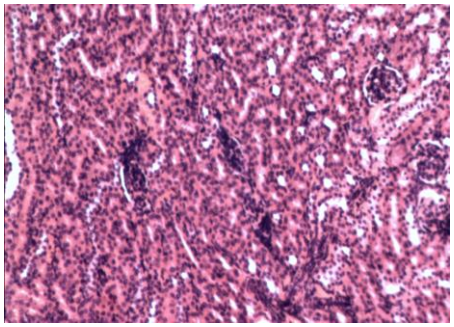
Control group



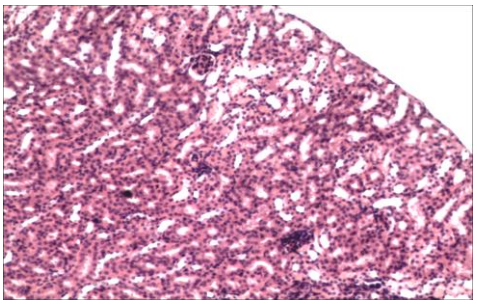
Model group



Berberine group



Baicalin group



Geniposide group

(d)

Fig. 5: The heart, liver, lung and kidney of the mice were harvested 24 h after inception of the experiment. Pathological investigation was performed following the treatment of formalin-fixed paraffin embedded and stained tissues. (a) The heart tissues of the mice. (b) The liver tissues of the mice. (c) The lung tissues of the mice. (d) The kidney tissues of the mice.

3.5 Neutralization of LPS in vivo

As described previously, the test of neutralization of LPS was carried out with the aid of Quantitative chromogenic tachypleus amebocyt lysati, allowing the detection of free LPS. The results indicated that the LPS levels in plasma was highest 2 h after intravenous injection and recovered to normal levels after 48 h. LPS levels of the medicated groups were lower than that of the model group (**p < 0.01). The medicated groups of berberine, baicalin and geniposide exhibited strong activity in neutralizing LPS. The results are shown in Table 3.

Table 3 The level of LPS in blood (n=10，$\bar{x}\pm s$)

Time	Model	Berberine	Baicalin	Geniposide
	EU/mL			
48h	3.89±0.09	3.34±0.06**	3.12±0.14**	3.41±0.06**
24h	5.50±0.12	4.01±0.09**	4.26±0.08**	4.43±0.05**
12h	5.54±0.07	4.36±0.04**	4.30±0.04**	4.50±0.04**
6h	6.13±0.14	5.21±0.16**	5.20±0.15**	5.59±0.07**
2h	7.22±0.06	7.06±0.04**	7.04±0.09**	6.07±0.02**

*p < 0.05 compared with model group; **p < 0.01 compared with model group.

The one-way analysis of variance (ANOVA) followed by Dunnett method was used

to analyze the data.

3.6 Suppression of TNF-α and IL-6 release

The activities of the medicated groups were tightly associated with the serum TNF-α and IL-6 levels; therefore, we tested the levels of TNF-α and IL-6 in animal models induced by LPS. In these experiments, it was found that mice that were treated with LPS had a large increase in TNF-α and IL-6, which peaked 2 h after LPS treatment. However, the peaks of TNF-α and IL-6 were reduced with the treatment of berberine, baicalin and geniposide (**$P < 0.01$). In particular, the medicated group of baicalin displayed significant activity on suppressing TNF-α and IL-6 release. The results are shown in Table 4-5.

Table 4 The release of IL-6 in blood (n=10，$\bar{x}\pm s$)

Time	Model	Berberine	Baicalin	Geniposide
	ng/L			
48h	203.9±4.5	170.1±3.0**	151.68±1.93**	160.50±1.7**
24h	280.6±8.4	203.5±1.0**	183.61±2.9**	185.81±1.35**
12h	284.5±6.3	205.2±2.9**	206.84±3.56**	207.74±2.61**
6h	338.5±8.3	290.56±1.8**	278.73±5.29**	310.19±1.24**
2h	463.8±7.4	322.83±2.05**	380.26±5.32**	353.37±7.98**

*$p < 0.05$ compared with model group; **$p < 0.01$ compared with model group.

The one-way analysis of variance (ANOVA) followed by Dunnett method was used to analyze the data.

Table 5 The release of TNF-α in blood (n=10，$\bar{x}\pm s$)

Time	Model	Berberine	Baicalin	Geniposide
	ng/L			
48h	101.53±2.3	95.29±1.39**	80.47±1.57**	83.53±1.95**
24h	151.76±2.42	120.91±2.17**	118.23±3.57**	121.46±1.69**
12h	160.24±1.49	126.50±1.74**	123.3±2.22**	121.25±2.01**
6h	181.18±3.24	134.93±1.45**	120.59±2.31**	136.13±1.64**
2h	279.88±1.69	230.71±1.59**	199.94±3.4**	240.44±1.21**

*$p < 0.05$ compared with model group; **$p < 0.01$ compared with model group.

The one-way analysis of variance (ANOVA) followed by Dunnett method was used to analyze the data.

4 Discussion

LPS is the major mediator of sepsis and research indicates that the molecules which bind to and neutralize LPS, could have potential anti-sepsis action (Wiese et al., 1999; Vallespi et al., 2003). Lipid A, the active center of LPS, has been identified as the toxic component of LPS and hence was used as an ideal target for screening anti-sepsis agents (Wyckoff et al., 1998; Wong et al., 2003). PMB, a well-established active compound that binds to lipid A, is a positively charged amphipathic cyclic oligopeptide linked to a single fatty acid, and has been shown to bind to lipid A stoichiometrically (1:1) with a *Kd* of approximately 10–100 nM (as assessed by different methods) (David et al., 1992; Ried et al., 1996). Thusly, PMB was used as a control in the experiment for assessing the binding affinity of lipid A and the components from HJD. The K_d values of berberine, baicalin and geniposide, which reflects the affinity between these compounds and lipid A, were obtained. Berberine, baicalin and geniposide displayed lower binding affinities to lipid A and higher K_d values compared to PMB. Research indicated that most lipid A $_D$-GlcpN-containing backbones are phosphorylated at positions 1 of the proximal a-GlcpN (GlcpN I) and 4′ of the distal b-GlcpN (GlcpN II) (Molinaro et al., 2015). After analysis of the structures of the three compounds, we speculated that the positively charge quaternary ammonium of the berberine bound to the negatively charged phosphoric acid of the disaccharide in lipid A (David et al., 1992; Ried et al., 1996). The hydroxyl groups of baicalin and geniposide can react with the phosphate groups in lipid A to form an ester (Trent et al., 2006). The present study takes PMB, which has been internationally recognized to have the ability to neutralize the activity of LPS, as an example to test the affinity of PMB to lipid A with the aid of a biosensor. The results indicate that there is high affinity between PMB and lipid A, which validates that a biosensor could be used to separate and extract the effective components of TCM for anti-LPS/lipid A. This study focuses on the selection of the effective components from HJD that can be used in future studies for anti-endotoxin purposes. Therefore, the anti-endotoxin activity of PMB has not been valued in the

present study. Although the affinity between berberin, baicalin and geniposide from HJD with lipid A accounts for 10% of the affinity for PMB, the three components all exhibited affinity with lipid A, which indicates that the three components function together to treat sepsis in HJD. Therefore, given the high affinities of berberine, baicalin and geniposide with lipid A, they were used for further *in vivo* experiments.

Sepsis results in the stimulation of numerous proinflammatory mediators such as TNF-α and IL-6, which may damage cells and lead to organ injury (Karima et al., 1999; Martin et al., 2003). Therefore, activity experiments were carried out to assess the levels of LPS, IL-6 and TNF-α. Model animals were injected with berberine, baicalin and geniposide to assess their activities. The LPS level in the model group was highest 2 h after the LPS injection and then decreased gradually. However, the LPS level was reduced in the three medicated groups of berberine, baicalin and geniposide. These studies indicate that berberine, baicalin and geniposide could neutralize LPS *in vivo*. The levels of IL-6 and TNF-α increased in the model group induced by LPS and peaked 2 h after the LPS injection. This study revealed that the three medicated groups could decrease the release of inflammatory factors IL-6 and TNF-α induced by LPS. These results suggest that berberine, baicalin and geniposide inhibit LPS activity on the activation of inflammatory cells *in vivo*. In addition, research shows that geniposide displays anti-inflammatory activity through inhibiting the activity of NF-κB and pro-inflammatory cytokine release in the TLR4-NF-κB pathway in macrophages treated with LPS (Huang et al., 2013). Berberine exhibits anti-inflammatory activity by inhibiting the NF-κB and MARK pathway and pro-inflammatory cytokine release in LPS-induced RAW264.7 cells (Yang et al., 2006). Baicalin inhibits the expression of TLR4 and the release of TNF-α (Yu et al., 2009). This suggests that berberine, baicalin and geniposide may inhibit the inflammatory factors released by neutralizing LPS and inhibiting the TLR4-NF-κB pathway. Pathological research shows that the model group exhibited different degrees of injury in the liver, heart, lung and kidney induced by LPS. The medicated treatment groups exhibited protective activity on varying organs. The group treated with berberine demonstrated stronger protective activity on the heart,

kidney and liver while geniposide displayed strong activity on the liver, heart and lung. Baicalin exhibited protective activity on the heart and liver. This suggested that berberine, baicalin and geniposide protect vital organs by inhibiting the proinflammatory mediators TNF-α and IL-6. Berberine is one of the main components of *Coptis chinensis Franch.* and *Phellodendron amurense Rupr,* Baicalin and geniposide are the principal components of *Scutellaria baicalensis Georgi* and *Gardenia jasminoides J.Ellis,* respectively. Based on the above mentioned research, it suggests that several components of HJD neutralize LPS and reduce the levels of IL-6 and TNF-α induced by LPS and protect vital organs. Mortality of patients who have suffered from septic shock is still 40%–60%, despite rapid progress in developing antibiotics and other therapeutic methods in clinical practice (Ohto et al., 2012). With the advantage of lower cost, faster curative effects and few side effects, HJD could be widely utilized as a drug in the treatment of LPS-induced diseases. Based on this, it is essential to figure out the mechanism of HJD for treating sepsis in order to provide a theoretical basis for further application in the treatment of diseases caused by LPS. Since the process of infection by LPS is complicated, there may exist many possible mechanisms of HJD for treating sepsis which we will further investigate. We believe that further studies on HJD will aid in our ability to design effective interventions for treating sepsis.

5 Conclusions

Berberine, baicalin and geniposide neutralize LPS by binding to lipid A and then reducing the release of IL-6 and TNF-α induced by LPS. Furthermore, berberine, baicalin and geniposide exhibit protective activity on varying organs compared to the animal models that were induced by LPS. This verifies that the components from HJD neutralize LPS and then reduce the release of IL-6 and TNF-α induced by LPS. HJD is an ideal treatment for sepsis due to multiple components of HJD having the ability to neutralize LPS, decrease the release of IL-6 and TNF-α induced by LPS, and protect vital organs.

Acknowledgments

This project was supported by the National Natural Science Foundation of China (No.81303205 and No.81303225) and program for Liaoning General Research (L2015330) and program for Liaoning Innovate Research Team in University (LT2013020). None of the authors declare any conflict of interests in this study.

References

Baker, R.R., Bishop, L.J., 2004. The pyrolysis of tobacco ingredients. J. Anal. Appl. Pyrolysis 71, 223-311.

Chen, L.J., Sun, X.M., Zhang, Z.W., et al., 2012. Comparison on fingerprints of extract solutions from Huanglian Jiedu Decoction by three kinds of methods. Chin. Herb Med. 3, 487-491. (In Chinese)

Dai, X., Gao, S., 2000. Experimental Study on anti-LPS effects of Huanglian Jiedu decoction in vitro and in vivo. Chinese J. Basic Med. Tradit. Chinese Med. 6, 31-32. (In Chinese)

David, S.A., Balasubramanian, K.A., Mathan, V.I., et al., 1992. Analysis of the binding of PMB to endotoxic lipid A and core glycolipid using a fluorescent displacement probe. Biochim. Biophys. Acta 1165, 147-152.

Diebold, S.S., Kaisho, T., Hemmi, H., et al., 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303, 1529-1531.

El-Naggar, L.J., Beal, J.L., 1980. Iridoids. A Review. J. Nat. Prod. 43, 649-707.

Fang, S., Qiu, Q., Hao, Y., 2001. Influence of Herbage Containing Serum of HuangLianJieDu Tang on LPS or TNF-α Induced Neutrophil Endothelium Cells Adhesion. Chinese J. Exp. Tradit. Med. Formulae 7, 31-33. (In Chinese)

Gattis, S.G., Chung, H.S., Trent, M.S., et al., 2013. The Origin of 8-Amino-3,8-dideoxy-D-manno-octulosonic Acid (Kdo8N) in the Lipopolysaccharide of Shewanella oneidensis. J. Biol. Chem. 288, 9216-9225.

Gao, B., Tsan, M.F., 2003. Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages. J. Biol.

Chem. 278, 174-179.

Genfa, L., Jiang, Z., Hong, Z., et al., 2005. The screening and isolation of an effective anti-endotoxin monomer from Radix Paeoniae Rubra using affinity biosensor technology. Int. Immunopharmacol. 5, 1007-1017.

Huang, X., Guo, L.H., Ma, S.Y., et al., 2012. Protective Effects of Huanglian Jiedu decoction on cardiac state in rats with sepsis. Chinese Journal of Integrative Medicine on Cardio-/Cerebrovascular Disease 6, 710-712. (In Chinese)

Huang, L.H., Wang, C.J., Naren, G.W., et al., 2013. Effect of geniposide on LPS-induced activation of TLR 4-NF-κB pathway in RAW264.7 macrophage cell line. Chin. J. Cell. Mol. Immunol. 29, 1012-1014. (In Chinese)

Jiang, Z., Hong, Z., Guo, W., et al., 2004. A synthetic peptide derived from bactericidal/permeability-increasing protein neutralizes endotoxin in vitro and in vivo. Int. Immunopharmacol. 4, 527-537.

Karima, R., Matsumoto, S., Higashi, H., et al., 1999. The molecular pathogenesis of endotoxic shock and organ failure. Mol. Med. Today 5, 123-132.

Li, J., Jiang, X., Li, H., et al., 2006. Experimental Study of the influence on the oral cavity bacteria colony and the bacteriolysin from Huanglian Jiedu Decoction as a Transdermal Therapeutant. J. Extern. Ther. Tradit. Chin. Med. 15, 7-9. (In Chinese)

Liu, P., Ye, H., Pan, J., et al., 2007. Inhibitory effect of the Huanglian Jiedu tang on β-lactamases-producing bacteria. Chinese J. Microecol. 19, 32-33. (In Chinese)

Ma, Y., Chen, L., Yu, Z., et al., 2000. Preliminary study on pharmacologic function of Huanglianjiedu Pilulae. Yunnan J. Tradit. Chinese Med. Mater. Medica 21, 35-36. (In Chinese)

Martin, G.S., Mannino, D.M., Eaton, S., et al., 2003. The epidemiology of sepsis in the United States from 1979 through 2000. N. Engl. J. Med. 348, 1546-1554.

Molinaro, A., Holst, O., Di Lorenzo, F., et al., 2015. Chemistry of Lipid A: At the Heart of Innate Immunity. Chemistry 21, 500-519.

Ohto, U., Fukase, K., Miyake, K., et al., 2007. Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa. Science 316, 1632-1634.

Ohto, U., Fukase, K., Miyake, K., et al., 2012. Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2. Proc. Natl. Acad. Sci. 109, 7421-7426.

Park, B.S., Song, D.H., Kim, H.M., et al., 2009. The structural basis of lipopolysaccharide recognition by the TLR4–MD-2 complex. Nature 458, 1191-1195.

Qin, M., Wang, H., 2003. Studies on Chemical constituents of Phellodendron Chinense Schneid Bark. Chem. Ind. For. Prod. 23, 42-46. (In Chinese)

Ried, C., Wahl, C., Miethke, T., et al., 1996. High Affinity Endotoxin-binding and Neutralizing Peptides Based on the Crystal Structure of Recombinant Limulus Anti-lipopolysaccharide Factor. J. Biol. Chem. 271, 28120-28127.

Smith, K.D., Andersen-Nissen, E., Hayashi, F., et al., 2003. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat. Immunol. 4, 1247-1253.

Takeuchi, O., Kawai, T., Mühlradt, et al., 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int. Immunol. 13, 933-940.

Trent, M.S., Stead, C.M., Tran, A.X., et al., 2006. Diversity of endotoxin and its impact on pathogenesis. J. Endotoxin Res. 12, 205-223.

Vallespi, M.G., Alvarez-Obregon, J.C., Rodriguez-Alonso, I., et al., 2003. A Limulus anti-LPS factor-derived peptide modulates cytokine gene expression and promotes resolution of bacterial acute infection in mice. Int. Immunopharmacol. 3, 247-256.

Wang, C., Cheng, H., Guan, Y., et al., 2007. Effect of Huanglian Jiedu Decoction on candida albicans biofilm formation in vitro. J. Trop. Dis. Parasitol. 5, 82-84. (In Chinese)

Wang, L.J., Xu, Q., 2000. Mechanism of anti-inflammatory action of

Huanglian Jiedu decoction a traditional Chinese prescription. Zhongguo Zhong Yao Za Zhi 25, 493 – 496. (In Chinese)

Wei, Y., Shan, L., Qiao, L., et al., 2013. Protective Effects of Huang-Lian-Jie-Du-Tang against Polymicrobial Sepsis Induced by Cecal Ligation and Puncture in Rats. Evid Based Complement Alternat Med. 2013, Article ID 909624.

Wiese, A., Brandenburg, K., Ulmer, A.J., et al., 1999. The dual role of lipopolysaccharide as effector and target molecule. Biol. Chem. 380, 767-784.

Wong, Y.N., Rossignol, D., Rose, J.R., et al., 2003. Safety, pharmacokinetics, and pharmacodynamics of E5564, a lipid A antagonist, during an ascending single-dose clinical study. J. Clin. Pharmacol. 43, 735-742.

Wyckoff, T.J., Raetz, C.R., Jackman, J.E., 1998. Antibacterial and Anti-inflammatory agents that target endotoxin. Trends Microbiol. 6, 154-159.

Xiao, C.L., Huang, H.Y., 2009. Clinical study on sepsis with the method of detoxification. Guide of China Medicine 26, 226-227. (In Chinese)

Yang, J., Wang, H.D., Lu, D.X., et al., 2006. Effects of neutral Sulfate berberine on LPS-reduced cardiomyocyte TNF-alpha secretion abnormal calcium cycling, and cardiac dysfunction. Acta Pharmacol. Sin. 27, 173-178.

Yu, D.T., Black, E., Sands, K.E., et al., Academic Medical Center Consortium Sepsis Project Working Group, 2003. Severe sepsis: variation in resource and therapeutic modality use among academic centers. Crit. Care. 7, R24-34.

Yu, M., Lei, L., Tan, W.X., et al., 2009. Effects of baicalin on Toll-like receptor 4 expression and tumor necrosis factor alpha secretion in human dental pulp cells induced by lipopolysaccharide. Chin. J. Conserv. Dent. 19, 632-636. (In Chinese)

Zhang, G., Ghosh, S., 2001. Toll-like receptor-mediated NF-κB activation: a

phylogenetically conserved paradigm in innate immunity. J. Clin. Invest. 107, 13-19.