



Extraction of the anti-sepsis component from Terminaliachebula Retz and evaluation of its biological activities

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Synopsis

Many clinical experiments and studies have demonstrated that traditional Chinese medicines possess the capacity for being used in anti-sepsis. In this paper, we screened 78 herbs based on biosensor technology by targeting of lipid A. Terminaliachebula Retz was found to possess the highest capability of binding lipid A. With CER (cation-exchange resin) and HPLC, we obtained three active components extracted from Terminaliachebula Retz, and named them TCR1, TCR2 and TCR3 respectively. These three components were evaluated with the biosensor, and it was found that the TCR3 was the most capable candidate to bind lipid A. We also studied the biological activities of TCR3 against sepsis *in vitro* and *in vivo*. *In vitro*, TCR3 could significantly inhibit LPS (lipopolysaccharide)-induced LAL (*Limulus* amoebocyte lysate) from agglutination and decrease TNF α (tumour necrosis factor α) release from RAW264.7 cells induced by LPS in a dose-dependent manner. *In vivo*, TCR3 could significantly protect mice against a lethal challenge with LPS and heat-killed *Escherichia coli* 35218 in a dose-dependent manner. These results demonstrate that Terminaliachebula Retz is an important herb to neutralize LPS and it has the potential to serve as a treatment for sepsis.

Key words: biosensor, lipid A, sepsis, Terminaliachebula Retz, tumour necrosis factor α (TNF α)

INTRODUCTION

The condition of sepsis can result in the activation of numerous pro-inflammatory mediators such as TNF α (tumour necrosis factor α), IL-6 (interleukin-6) and IL-12. It may, furthermore, cause the MODS (multiple organ dysfunction syndrome) [1,2]. In 1990, the CDC (Centres for Disease Control) estimated that there were 450 000 cases of sepsis per year in the United States, including 100 000 deaths, and CDC warned that the incidence had increased significantly despite the improvement in the management of septic patients with systemic antibiotics, surgical intervention, aggressive fluid resuscitation and careful monitoring [3,4]. The mechanism of biological activity of LPS (lipopolysaccharide) may involve specific binding of lipid A. As an evolutionarily conserved region of LPS, lipid A has been identified as the toxic component of LPS and hence represents an ideal target for anti-sepsis drug development [5]. Some substances, such as PMB (polymyxin B), BPI (bactericidal/permeability increasing

protein) and LALF (*Limulus* anti-LPS factor), can bind to lipid A and neutralize the toxicity of LPS. However, these substances cannot serve as a medicine for the treatment of sepsis. That is because PMB possesses severe nephrotoxicity; BPI at certain concentrations can prevent this activation of anti-LPS and it is difficult to obtain LALF of safe quality. In China, herbs have been used as traditional remedies for hundreds of years, and they have been found capable of treating more and more diseases. For example, acanthopanax root is used in the treatment of insomnia, the Chinese herb golden thread is used as a treatment for diarrhoea and so on. In recent years, studies have suggested that traditional Chinese herbs have played important roles in anti-sepsis treatment [6–10]. We thus became interested in exploring one of the Chinese herbs that could bind to lipid A and neutralize the toxicity of LPS. In the present study, we have analysed the biological activities of the component, which is screened and extracted from a traditional Chinese herb using affinity technology and HPLC, against sepsis. To the best of our knowledge, the present study is the first demonstration that Terminaliachebula

Abbreviations used: BPI, bactericidal/permeability increasing protein; CER, cation-exchange resin; CFU, colony-forming unit; DMEM, Dulbecco's modified Eagle's medium; IL-6, interleukin-6; LAL, *Limulus* amoebocyte lysate; LB, Luria-Bertani; LPS, lipopolysaccharide; LALF, *Limulus* anti-LPS factor; PMB, polymyxin B; TNF α , tumour necrosis factor α ; TLR4, Toll-like receptor 4.

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Retz, a traditional Chinese herb, possesses biological activities against sepsis.

MATERIALS AND METHODS

Cell line and culture

Murine macrophage RAW264.7 cells were purchased from the A.T.C.C. (Manassas, VA, U.S.A.) and maintained in DMEM (Dulbecco's modified Eagle's medium) that contains 10% low-endotoxin fetal calf serum (HyClone, Logan, UT, U.S.A.), 2 μ M glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin.

Bacterial strains and LPS

Bacterial strains of *Escherichia coli* ATCC 25922 were maintained in an LB (Luria–Bertani) agar plate, transferred to a sterile liquid LB medium (containing 10 g of tryptone, 10 g of NaCl and 5 g of yeast extract per litre) and cultivated aerobically in 50 ml volume at 37°C in a heated, shaking environmental chamber. When bacteria were in the exponential phase of growth, they were harvested. LPS (0111, B4; Sigma) was prepared as 2 mg/ml with normal saline.

Animals

A total of 160 KM mice, an equal number of males and females, were obtained from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). The average weight of the mice was 18–22 g. The mice were maintained in SPF (specific pathogen free) conditions until used. All experiments were conducted in accordance with the National Guidelines for the Care and Use of Laboratory Animals.

Screening herbs with biosensor technology

Lipid A was immobilized on the surface of a hydrophobic cuvette according to the manufacturer's instructions (Thermo Labsystems). The ground herbs were soaked in water for 24 h and then boiled in water at 100°C for 45 min after washing thoroughly with distilled water. After filtration, the material was centrifuged at 4000 g for 30 min and the supernatants were collected and are termed 'aqueous extract' here. A 5 μ l portion of aqueous extract (15 g/l) from each herb was added to a cuvette containing 60 μ l of PBS. After 5 min, the cuvette was washed seven times with 60 μ l of PBS and alternately washed with 0.1 M HCl, PBS and 10 mM NaOH respectively. Data analysis was performed with the FASTplot software package (Thermo Labsystems).

Isolation with CER (cation-exchange resin) and HPLC

An aqueous extract subjected to CER-SO₃H (where SO₃H is a sulfonic group) purchased from Qingdao Marine Chemical Fact-

ory (Qingdao, China) was eluted with 20% ammonia solution and 80% dehydrated alcohol. The eluate was collected and concentrated by rotary evaporation (BUCHI Rotavapor R205). The HPLC system was balanced with 0.1 mol/l HCl solution (pH 3). The eluate was then injected on to the HPLC system (Agilent) and eluted with 0–100% mol/l NaCl to yield three HPLC components, namely TCR1, TCR2 and TCR3 (Figure 1).

The Limulus test

The Limulus test is a new *in vitro* organism detection technology, which utilizes the gelatination reaction between LAL (*Limulus* amoebocyte lysate) and a microamount of endotoxin to detect the concentration of a microamount of endotoxin with dynamic nephelometry. Different concentrations of the TCR3 (10, 20, 40 and 80 μ g/ml) were incubated respectively with LPS (10 ng/ml) at 37°C for 30 min. PMB (1 μ g/ml) served as the positive control and 10 ng/ml LPS served as the negative control. A 100 μ l portion of this mixture was added to LAL reagent of equal volume. The kinetic turbidity was measured using an EDS-99 tube reader (Zhanjiang A&C Biological) (Figure 3).

Cytokine release induced by LPS

RAW264.7 cells (0.5 ml) were incubated in a 96-well plate for 4 h, and the supernatants were then discarded and replaced with 0.5 ml of serum-free DMEM. The cells were pretreated with TCR3 at the indicated dose (10, 20, 40 and 80 μ g/ml) for 30 min and then stimulated with LPS (100 μ g/l) for 4 h. The supernatants were collected to assess the TNF α level using the appropriate ELISA kits. LPS+PMB (1 μ g/ml) served as the positive control and TCR3 (80 μ g/l) served as the blank (Figure 4).

Animal protection attacked by LPS

A total of 80 mice were randomly divided into four groups (20 mice per group) and intravenously injected as follows: LPS (20 mg/kg) alone in the first group, TCR3 (50 mg/kg) in the second group as the medical blank, LPS (20 mg/kg) and immediate subsequent injection of TCR3 (50 mg/kg) as the treatment in the third group, LPS (20 mg/kg) and immediate subsequent injection of PMB (1 mg/kg) as the positive control in the fourth group. The general conditions and mice mortalities were observed at 6, 12, 24, 36, 48, 60, 72 and 96 h respectively after injection (Figure 5).

Animal protection attacked by heat-inactivated *E. coli*

A total of 80 mice were again randomly divided into four groups (20 mice per group) and intravenously injected as follows: *E. coli* [1.2×10^{10} CFU (colony-forming units)/kg] alone in the first group, TCR3 (50 mg/kg) in the second group as the medical blank, *E. coli* (1.2×10^{10} CFU/kg) and immediate subsequent injection of TCR3 (50 mg/kg) as the treatment in the third group, LPS (20 mg/kg) and immediate subsequent injection of

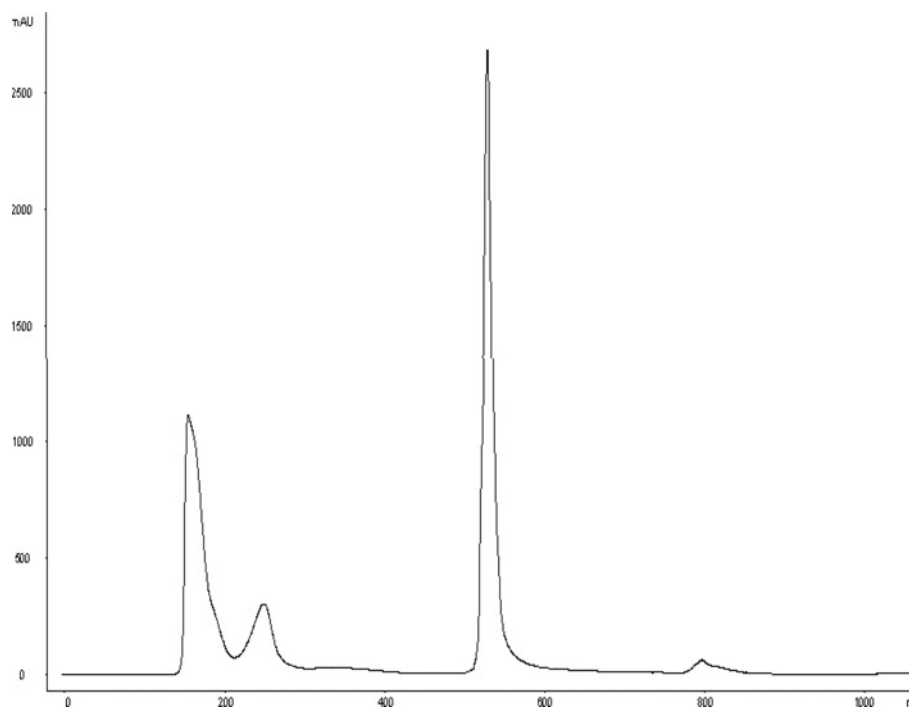


Figure 1 Isolation of *Terminaliachebula Retz* by ion-exchange HPLC

The HPLC system was balanced with 0.1 mol/l hydrochloric acid solution (pH 3). The eluate collected by CER-SO₃H was then injected on to the HPLC system (Agilent) and eluted with 0–100% mol/l NaCl. Three components were isolated from *Terminaliachebula Retz* by ion-exchange HPLC: TCR1, TCR2 and TCR3. Of these, TCR3 has the highest binding ability to lipid A.

dexamethasone (5 mg/kg) as the positive control in the fourth group. The general conditions and mice mortalities were observed at 6, 12, 24, 36, 48, 60, 72 and 96 h respectively after injection (Figure 6).

Statistical analysis

Results are expressed as the means $\pm$ S.D. The Student's *t* test was used to examine the differences between the groups. $P < 0.05$ (double-sided) was considered significant and $P < 0.01$ was considered very significant.

RESULTS

Biosensor technology was performed to screen the 78 traditional Chinese herbs. The 78 chosen herbs all had anti-inflammatory ability according to the drug lexicon. Out of the 78 herbs, 12 were identified to have higher binding activities; *Terminaliachebula Retz* was identified to have the highest binding activity to lipid A of the 12 herbs. Using CER and HPLC, three components were extracted from *Terminaliachebula Retz* and named as TCR1, TCR2 and TCR3 respectively (Figure 1). Based on biosensor technology, TCR3 was found to have an outstanding

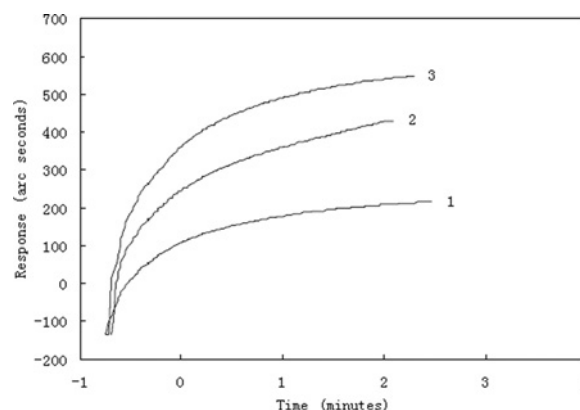


Figure 2 Binding ability to lipid A of the three components isolated from *Terminaliachebula Retz*

The binding ability to lipid A was determined with biosensor technology. Lipid A was immobilized on the surface of a hydrophobic cuvette according to the manufacturer's instructions (Thermo Labsystems). The binding was determined by the optic principle. Note that the single angle can be extremely sensitive to the physical parameters of the system; any small change in the refractive index n_1 results in a relatively large change in the angle θ_{guide} . These refractive index changes are a result of binding. Line 3, TCR3; line 2, TCR1; line 1, TCR2.

binding activity to lipid A (Figure 2). To further evaluate the biological activities of TCR3 against sepsis, experiments were performed *in vitro* and *in vivo*. The TCR3 showed significant

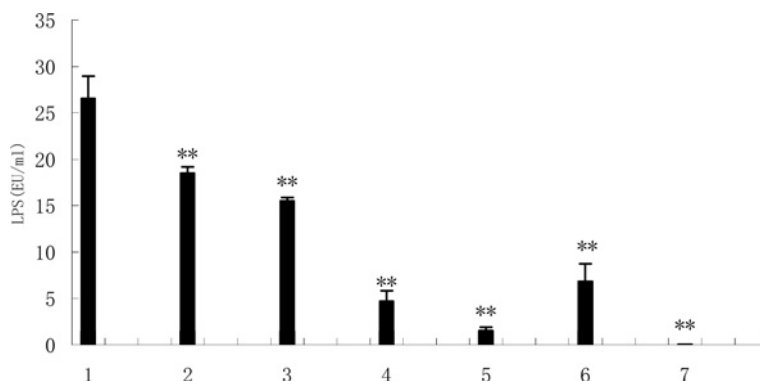


Figure 3 Neutralization by TCR3 of LPS with the Limulus test

Neutralization by TCR3 of LPS *in vitro* is shown. Indicated concentrations of TCR3 were incubated with 0.1 µg/l LPS at 37°C for 30 min. Subsequently, 100 µl of this mixture was added to an equal volume of the LAL reagent and the kinetic turbidity was measured. The test was repeated three times. The numbers 1, 2, 3, 4, 5, 6 and 7 represent LPS (10 ng/ml), LPS+TCR3 (10 µg/ml), LPS+TCR3 (20 µg/ml), LPS+TCR3 (40 µg/ml), LPS+TCR3 (80 µg/ml), LPS+PMB (1 µg/ml) and the medical blank respectively. Each test was repeated three times. ** $P < 0.01$ compared with LPS without TCR3. EU, endotoxin unit.

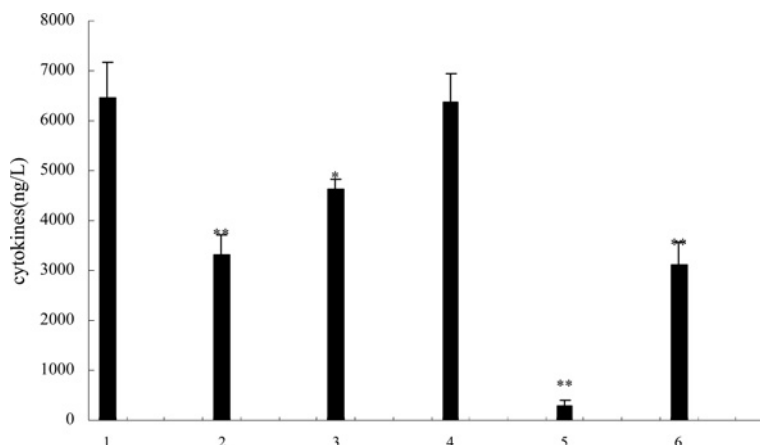


Figure 4 Inhibition of TNFα secretion in RAW264.7 cells by TCR3

The effect of TCR3 on cytokine secretion from RAW264.7 cells induced by LPS is shown. RAW264.7 cells (0.2 ml) were pre-incubated with indicated concentrations of TCR3 for 30 min and then stimulated with LPS (100 µg/l). After the cells were incubated for another 4 h, the levels of TNFα in the supernatants were analysed using the appropriate ELISA kits. Cytokine levels are expressed as means $\pm$ S.D. in ng/l. A Student's *t* test was used to examine the differences. The numbers 1, 2, 3, 4, 5 and 6 represent LPS (100 ng/ml), LPS+TCR3 (80 µg/ml), LPS+TCR3 (40 µg/ml), LPS+TCR3 (20 µg/ml), the medical blank and LPS+PMB (10 µg/ml) respectively. Each test was repeated three times. * $P < 0.05$, ** $P < 0.01$ compared with LPS without TCR3.

LPS-neutralizing effect *in vitro* (Figure 3) and it could significantly inhibit the TNFα release in RAW264.7 cells induced by LPS (Figure 4). The TCR3 also protected the mice from lethal challenge of LPS or heat-inactivated *E. coli* *in vivo* (Figures 5 and 6).

DISCUSSION

Sepsis, septic shock and the subsequent multiple organ failure are among the most common causes of death in intensive care units

[1]. The incidence of sepsis and septic shock has increased significantly despite the improvement in the management of septic patients with systemic antibiotics, surgical intervention, aggressive fluid resuscitation and careful monitoring [2–4]. As the major part of the extracellular membrane of Gram-negative bacteria, LPS is recognized as a key molecule in the pathophysiology of sepsis. LPS can bind to its receptors in the cell membrane through its active region – lipid A, and then induce sepsis. Thus, the blocking of the binding of lipid A to the receptors is considered the most promising strategy to prevent the initiation of sepsis [11]. Although clinical experiments and studies have suggested that many traditional Chinese medicines possess capacity for being

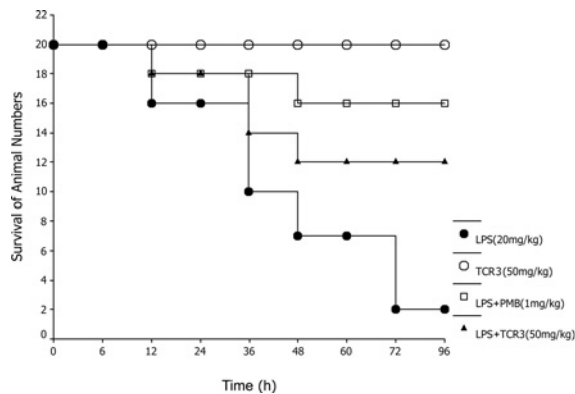


Figure 5 Protective effect of TCR3 treatment on the animals challenged with a lethal dose of LPS

The protective effect of TCR3 on the survival rate of mice challenged with a lethal dose of LPS is shown. Mice were randomly divided into four groups ($n=20$): LPS (20 mg/kg) alone in the first group, TCR3 (50 mg/kg) in the second group as the medical blank, LPS (20 mg/kg) and an immediate subsequent injection of PMB (1 mg/kg) as the positive control in the third group and LPS (20 mg/kg) and an immediate subsequent injection of TCR3 (50 mg/kg) as the treatment in the fourth group. The general conditions and mice mortalities were observed at 6, 12, 24, 36, 48, 60, 72 and 96 h respectively.

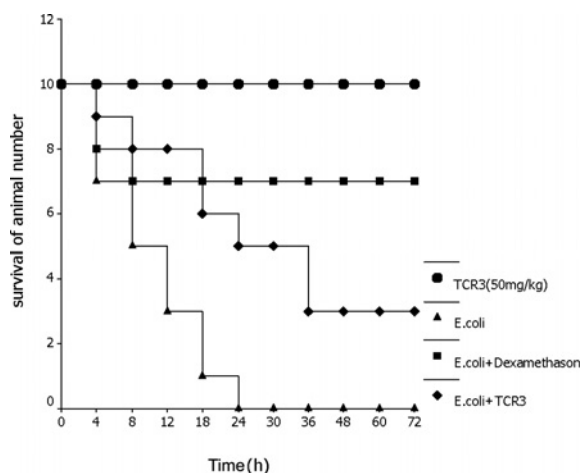


Figure 6 Protective effect of TCR3 treatment on animals challenged with a lethal dose of heat-inactivated *E. coli*

The protective effect of TCR3 on the survival rate of mice challenged with a lethal dose of heat-inactivated *E. coli* is shown. Mice were randomly divided into four groups ($n=20$): TCR3 (50 mg/kg) in the first group as the medical blank, *E. coli* (1.2×10^{10} CFU/kg) alone in the second group, LPS (20 mg/kg) and immediate subsequent injection of dexamethasone (5 mg/kg) as the positive control in the third group and *E. coli* (1.2×10^{10} CFU/kg) and an immediate subsequent injection of TCR3 (50 mg/kg) as the treatment in the fourth group. The general conditions and mice mortalities were observed at 4, 8, 12, 18, 24, 30, 36, 48, 60 and 72 h respectively.

used in anti-sepsis [6–11], there are many complicated chemical constituents in these herbs, and the material basis of the anti-sepsis activity is therefore not clear. Based on biosensor technology by targeting of lipid A, we screened the active components extracted

from Terminaliachebula Retz and studied their biological activities against sepsis.

In the present experiments, Terminaliachebula Retz was screened with biosensor technology from 78 herbs and then isolated with HPLC. Terminaliachebula Retz has been identified to have the highest binding activity to lipid A, a conserved region of LPS. Because lipid A takes positive charge, the component of Terminaliachebula Retz with positive charge processed using CER is eluted. With HPLC, the eluate was separated into three fractions, namely TCR1, TCR2 and TCR3 respectively. TCR3 was then identified to have the highest binding ability to lipid A among all three components. We also investigated the biological activities against sepsis with active components extracted from Terminaliachebula Retz *in vitro* and *in vivo*. *In vitro*, the TCR3 could neutralize LPS and inhibit cytokine (TNF α) release significantly. *In vivo*, animals were injected with a lethal dose of LPS or heat-inactivated *E. coli* and subsequently injected with TCR3 as a treatment. We observed the death count at the times indicated in Figures 5 and 6 and found that TCR3 could predominantly protect the mice from death. The concentration of TCR3 is relatively high, because a lethal dose of LPS was used in this experiment. Animals injected with TCR3 (50 mg/kg) alone were unaffected and had no fatality, which demonstrated that TCR3 does not possess obvious side effects and toxicity. This result suggested that Terminaliachebula Retz has the effect of neutralizing LPS, which could be explained since TCR3 competitively combines with TLR4 (Toll-like receptor 4), a pattern recognition receptor for LPS. The combination inhibits the TLR4-mediated signal transduction pathway to reduce the release of cytokines. Therefore we believe that Terminaliachebula Retz has the potential to serve as a medicine for the treatment of sepsis.

Conclusions

To the best of our knowledge, the present study is the first to report that Terminaliachebula Retz, a traditional Chinese herb, possesses biological activities against sepsis. These results suggest that Terminaliachebula Retz is an important herb to neutralize LPS and it may potentially serve as a medicine for the treatment of sepsis.

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