



2004

Chemical identification on the origins of phellodendri cortex

Follow this and additional works at: <https://www.jfda-online.com/journal>

Recommended Citation

Juang, L.-J. and Sheu, S.-J. (2004) "Chemical identification on the origins of phellodendri cortex," *Journal of Food and Drug Analysis*: Vol. 12 : Iss. 1 , Article 7.
Available at: <https://doi.org/10.38212/2224-6614.2666>

This Original Article is brought to you for free and open access by Journal of Food and Drug Analysis. It has been accepted for inclusion in Journal of Food and Drug Analysis by an authorized editor of Journal of Food and Drug Analysis.

Detection of Endotoxin Contamination in Chinese Herbs by NF- κ B Activity-Based Reporter Assays

YA-HUI HSU^{1,2} AND SHU-LING FU^{1*}

¹ Institute of Traditional Medicine, National Yang-Ming University, 155, Sec. 2, Linong St., Beitou District, Taipei City 112, Taiwan (R.O.C.)

² Bureau of Food and Drug Analysis, Department of Health, Executive Yuan, 161-2 Kunyang St., Nangang District, Taipei City 115, Taiwan (R.O.C.)

(Received: May 27, 2003; Accepted: August 12, 2003)

ABSTRACT

Severe LPS contamination in drugs and food can cause health problems and occasionally mislead research conclusions. To ensure the quality of traditional Chinese medicinal herbs (TCMH), LPS contamination problem should be evaluated. We here described a NF- κ B activity-based reporter assay to detect LPS contamination. We first created a macrophage cell line with integrated reporter gene consisting of NF- κ B-responding sites upstream of the *luciferase* gene. The presence of LPS leads to NF- κ B activation, and thus triggers downstream luciferase expression. The specificity of LPS-derived luciferase activity was confirmed by adding the LPS inhibitor, polymyxin B. In our system, the level of LPS correlates well with luciferase activity. The LPS activity is completely inhibited by polymyxin B, and the limit of LPS detection is 1ng/mL. We also utilized RT-PCR to demonstrate that LPS contamination at the concentration of 1ng/mL was enough to induce expression of downstream inflammatory cytokines TNF- α and IL-6. We further applied the method to examine LPS contamination in TCMH. Among the 35 herbal extracts we examined, about 20% of them exhibited variable but detectable LPS contamination (higher than 1ng/mL). The data indicate that LPS contamination in Chinese herbs should be considered. In addition, the ease and low-background feature of this assay suggest its potential application for systematic detection of LPS contamination in Chinese herbs.

Key words: LPS, endotoxin, NF- κ B, herbs, cytokine, reporter assays

INTRODUCTION

Traditional Chinese medicinal herbs (TCMH) had been well accepted for medical treatment and food supplements for many centuries. The quality control of TCMH is very important. In addition to evaluate authenticity of TCMH, efficacy of major components, contamination of adulterated chemicals and microbial contamination in TCMH should also be considered. Indeed, microbial contamination of TCMH might occur during growth, collection, preservation and drug manufacturing process. Although microorganisms themselves can be destroyed by prolonged heating, certain heat-resistant toxic derivatives of microorganisms, such as lipopolysaccharides (LPS; also known as endotoxin), might still exist.

LPS is a major and essential integral component of outer membrane of Gram-negative bacteria. LPS is an efficient microbial stimulant of innate immune response. By inducing the release of inflammatory cytokines, LPS activates both innate and adaptive immune responses, but excessive amount of cytokines by LPS induction can trigger fatal septic shock and sepsis in some circumstances⁽¹⁾. Patients suffering from sepsis exhibit systemic inflammation, followed by hypotension, multi-organ failure and death. TNF- α is regarded as a central mediator of the pathophysiological changes associated with LPS. However,

many cytokines such as IL-1 and IL-6 are also important mediators of sepsis⁽²⁾. The molecular mechanism(s) by which LPS initiate cytokine expression have recently been delineated. Macrophages play a pivotal role in LPS-induced effects. LPS first binds to lipopolysaccharide binding protein (LBP) to form LPS-LBP, which subsequently form a complex with a membrane protein called CD14. The LPS-LBP-CD14 complexes further interact with Toll-like receptor 4 (Tlr4) on the cell surface of the macrophages, which exclusively transduce the LPS signal across the membrane^(3,4). Activation of Tlr4 initiates a signaling pathway, leads to the activation of the transcription factor NF- κ B, and triggers production of pro-inflammatory cytokines (e.g. TNF- α , IL-1, IL-6)⁽⁵⁾.

In our recent experiments using medicinal herbs or herb-derived crude extracts, we found some of these herbal extracts were LPS-contaminated, as determined by chromogenic *Limulus* amoebocyte lysate (LAL) assays. The LPS contamination in these herbs significantly interfered with our results. Indeed, several documents have revealed that endotoxin contamination of samples can give false results and mislead conclusions^(6,7). Since severe LPS contamination can cause health problems and mislead research conclusions, endotoxin contamination of TCMH should be carefully scrutinized. Currently, the most common method to measure LPS is chromogenic LAL assay. This assay is quantitative and sensitive, but has some limitation for analyzing herbal extracts (for details, please see discus-

* Author for correspondence. Tel: 886-2-28267177; Fax: 886-2-28225044; Email: slfu@ym.edu.tw

sion). For example, color derived from herbal extracts may interfere with the spectrophotometric analysis of the assay.

Based on the finding that activation of Tlr4 by LPS could cause NF- κ B activation in cultures⁽⁸⁾, transfection-based reporter assays have been applied to analyze LPS-signaling pathways^(9,10). In these studies LPS-responsive cells were created by transfection with a reporter gene, which is capable of detecting NF- κ B activity, in a Tlr4-expressing cell type. In this paper, we successfully adapted this methodology to establish a NF- κ B activity-based reporter assay to detect LPS in TCMH. We first created a highly LPS-responsive macrophage cell line with stable integration of the reporter plasmid, which contains NF- κ B-responsive element followed by *luciferase* gene. The presence of LPS therefore can be reflected by luciferase activity. To examine whether the luciferase activity is truly resulted from LPS, we tested whether LPS-specific inhibitor polymyxin B can abolish luciferase activity. We also applied the method to examine LPS contamination in 35 herbal extracts.

MATERIALS AND METHODS

I. Reagents and Materials

Lipopolysaccharide (LPS; *Escherichia coli* serotype 0111:B4) and polymyxin B sulfate were purchased from Sigma (St. Louis, USA). Traditional Chinese herbs were purchased from herbal stores in Taipei. Twenty-six types of raw materials include *Achyranthes bidentata*, *Asparagus cochinchinensis*, *Astragalus membranaceus*, *Angelica sinensis*, *Bupleurum falcatum*, *Broussonetia papyrifera*, *Cornus officinalis*, *Cistanche salsa*, *Dioscorea batatas*, *Davallia formosana*, *Eleutherococcus senticosus*, *Ganoderma lucidum*, *Glycyrrhiza uralensis*, *Lithospermum erythrorhizon*, *Leonurus heterophyllus*, *Ledebouria seseloides*, *Ligusticum chuanxiong*, *Ophiopogon japonicus*, *Poria cocos*, *Panax ginseng*, *Paeonia lactiflora*, *Panax pseudoginseng*, *Polystictus versicolor*, *Rehmannia glutinosa*, *Schisandra chinensis* and *Scutellaria baicalensis*. Three types of concentrated herbal extracts include *Artemisia capillaries*, *Bupleurum falcatum* and *Rubus chingii*. Six types of partially purified fractions include *Agaricus blazei*, *Antrrodia cinamome*, *Cordyceps sinensis*, *Graptopetalum paraguayense*, *Taraxacum mongolicum* and *Polystictus versicolor*.

II. Preparation of Reagents or Herbal Extracts from Drugs or Medicinal Herbs

One gram of powdered herbs (raw materials, concentrated herbal extracts or partially purified fractions) were re-suspended in 10 mL of sterile deionized water, stirred overnight at 4°C, and sterilized through 0.22 μ m filter (Millipore, Billerica, USA). LPS (500 μ g) was dissolved in 500 μ L of PBS as stock solution (1mg/mL). Ten mg of polymyxin B sulfate was re-suspended in 1 mL of sterile

deionized water. Both LPS and polymyxin B stock solutions were sterilized by filtration. Undetectable LPS contamination in PBS or sterile deionized water was confirmed by chromogenic LAL assays QCL-1000 (Bio Whittaker, Walkersville, USA).

III. Cells and Medium

Murine macrophage-like cell line RAW 264.7 was purchased from Food Industry Research and Development Institute (Hsinchu, Taiwan). DMEM media were purchased from Biochrom KG (Berlin, Germany), and bovine calf serum (BCS) was from HyClone (Logan, USA). Antibiotics (penicillin and streptomycin) and sodium pyruvate were purchased from Invitrogen (Carlsbad, USA). RAW 264.7 cells were regularly cultured at 37°C in 5% CO₂ incubator in DMEM supplemented with 10% heat-inactivated BCS, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 1 mM sodium pyruvate.

IV. Creation of LPS-Responsive Cells with Stably Integrated Reporter Gene

RAW 264.7 cells (6×10^6) were seeded in 100-mm plates the day before transfection. Ten μ g of reporter plasmid pELAM1-Luc and 2 μ g of marker plasmid pCI-puro were co-transfected into cells using FuGENE 6 method according to manufacturer's instructions (Roche Inc., Basel, Switzerland). The pELAM1-Luc consists of NF- κ B-containing region from ELAM1 (endothelial leukocyte adhesion molecule I), followed by the reporter gene firefly *luciferase*. The plasmid pCI-puro directs expression of the puromycin-resistant gene. Forty-eight hr after transfection, the medium was replaced with puromycin-supplemented medium (4 μ g/mL) and continually selected for the puromycin-resistant cell clones. Three batches of puromycin-resistant cells were collected and screened for their responsiveness to LPS by luciferase assays as described below.

V. Luciferase Assays

LPS-responsive cells were seeded in MP-24 plates at the density of 2×10^5 cells per well. After treatment with LPS or drugs for 5 hr in 5% CO₂ incubator at 37°C, cells were harvested and lysed in 100 μ L of lysis reagent. Twenty μ L of cell lysate was then mixed with 100 μ L of luciferin (the substrate of luciferase) right before luminescence detection. The luminescence, generated by luciferase activity, was measured with AutoLumat LB953 (Berthold technologies, Bad Wildbad, Germany). All reagents for luciferase assays were purchased from Promega (Madison, USA).

VI. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from drug or herb-treated RAW

264.7 cells using Trizol reagent (Invitrogen Inc., Carlsbad, USA) according to the manufacturer's instructions. Total RNA (0.1 or 1 μg) was reverse-transcribed and amplified using Ready To GoTM (Amersham Biosciences, Uppsala, Sweden). The primer sequences are as follows: (1) mouse IL-6: sense, GGCAGAGTCCTTCAGAGAGAGATACAG; antisense, CCAACATTCATATTGTCAG; (2) mouse TNF- α : sense, AACTTCGGGGTGATCGGTCC; antisense, CAAATCGGCTGACGGTGTGGG; (3) mouse GADPH: sense, TGTGATGGGTGTGAACCACGAG; antisense, TGCTGTTGAAGTCGCAG GAGAC. Expected PCR products for IL-6, TNF- α and GADPH are 343, 284 and 471 base pairs, respectively. Thirty amplification cycles were performed for TNF- α and GADPH (0.1 μg of total RNA; PCR: 94°C for 1 min, 58°C for 1 min and 72°C for 1 min). Thirty-five cycles were performed for IL-6 (1 μg of total RNA; same PCR condition as TNF- α). PCR products were analyzed with 2% agarose gels stained with ethidium bromide.

VII. Statistical Analysis

Data are presented as mean values \pm SEM. Data were analyzed by one-way ANOVA for multiple comparisons using a commercially available statistical analysis program (SPSS, Arlington, VA). Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The procedure to establish LPS detection system is outlined in Figure 1. We first transfected the murine macrophage RAW 264.7 (with endogenous Tlr4 receptor)

with pELAM1-Luc plasmid, which contains the NF- κ B-responsive region of ELAM-1 (endothelial leukocyte adhesion molecule I) followed by the firefly *luciferase* gene. Transfected cultures were then subjected to puromycin selection. To screen for cells with integrated reporter plasmid pELAM1-Luc, different batches of puromycin-resistant cells were further tested for their responses to LPS. Following LPS treatment, NF- κ B proteins presumably will translocate into nucleus, bind to NF- κ B-binding sites on pELAM1-Luc DNA, and trigger expression of *luciferase* gene. Namely, luciferase activity corresponds to LPS-induced NF- κ B activity. After exogenously adding luciferin to cell lysates, the luciferase-luciferin reactions generate luminescence with high sensitivity and can be easily measured. Luciferase activity is directly proportional to total luminescence. Since there is no luciferase homolog in mammalian cells, the luciferase activity in mammalian cells is generally undetectable, which gives rise to the low background signal in this assay. The batches of cells exhibiting the highest luciferase responses to LPS were chosen for subsequent experiments. These cells are designated as RAW 264.7/Luc cells in the subsequent paragraphs.

The NF- κ B response curve for different amount of LPS is shown in Figure 2. The LPS response is dosage-dependent in luciferase assays; the luciferase activity increases when LPS concentrations increase. The limit of detection is 1 ng/mL. Figure 2 also demonstrates the LPS response can be inhibited by polymyxin B, a known pharmacological antagonist of LPS^(11,12). Effect of LPS at concentrations up to 300 ng/mL was completely abolished by polymyxin B at a concentration of 10 $\mu\text{g/mL}$. To determine whether the LPS response detected in our assays correlated

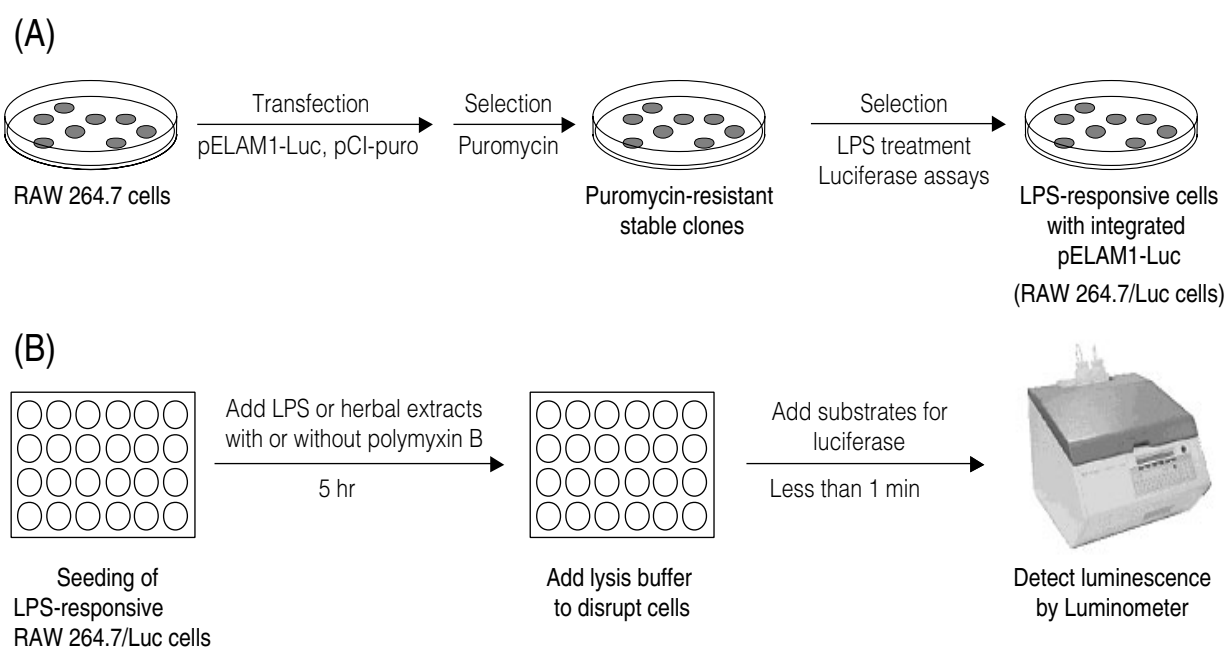


Figure 1. The protocol to establish LPS detection system. (A) Creation of LPS-responsive clones with integrated *luciferase* gene. (B) Detection of LPS contamination by NF- κ B-based luciferase assays.

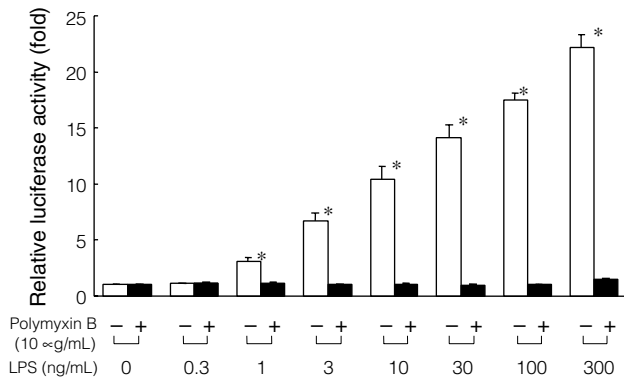


Figure 2. LPS specifically induces NF- κ B activation in a dose-dependent manner. The RAW 264.7/Luc cells were treated with increasing amount of LPS or pre-mixed LPS-polymyxin B for 5 hr. Data shown are the mean and SEM of three independent experiments, and are presented as fold increase over control cells (vehicle only). “*” represents $p < 0.05$ as compared with control (vehicle).

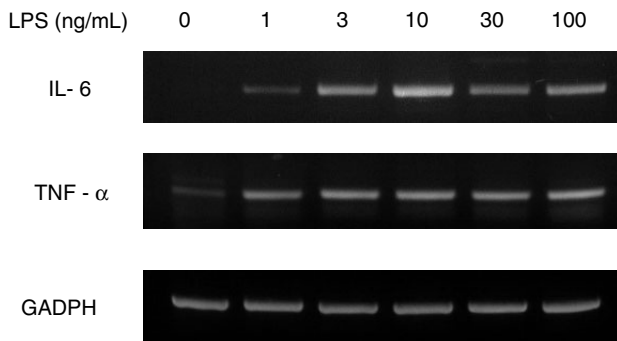


Figure 3. Induction of pro-inflammatory cytokine expression by increasing concentrations of LPS. Data shown are electrophoretic analysis of RT-PCR products for IL-6, TNF- α and internal control GAPDH. The expected sizes are 343 base pairs for IL-6, 284 base pairs for TNF- α and 471 base pairs for GAPDH, respectively.

with biological effects, we utilized RT-PCR techniques to analyze the expression of downstream pro-inflammation cytokines, such as TNF- α and IL-6. As shown in Figure 3, the presence of LPS at the concentration as low as 1 ng/mL was enough to induce expression of TNF- α and IL-6. An approximate dosage-dependent expression profile was observed. Therefore, we have established a reporter assay to detect LPS effects, and the LPS response detected by our assay can reflect its biological effects, as shown by cytokine expression.

We further applied this assay, in the presence or absence of polymyxin B, to detect LPS contamination in TCMH. We had examined a total of 35 types of aqueous extracts of TCMH, including 26 raw materials, 3 concentrated extracts and 6 partially purified fractions. Herbal extract (1 mg/mL) or partially purified fraction (0.3 mg/mL) was assayed in the absence or presence of polymyxin B (10 μ g/mL). Among them, water extracts from 5 raw materials, 1 concentrated extract and 2 partially purified extracts exhibited statistically significant LPS activity as compared with the control sample (vehicle only). Selected representative examples are shown

in Figure 4. The LPS-dependent activities of these extracts were confirmed by their suppression by polymyxin B (10 μ g/mL). We did exclude *C. sinensis*, since it still exhibits relatively high luciferase activity after polymyxin B treatment. To determine whether *C. sinensis* extract contains excessive LPS which cannot be completely neutralized by polymyxin B (10 μ g/mL), we treated the *C. sinensis* extract with higher amount of polymyxin B (20 μ g/mL). The luciferase activity of *C. sinensis*-treated cells by polymyxin B treatment at both concentrations showed no significant difference (data not shown), suggesting there exists LPS-independent luciferase activity in *C. sinensis*. The LPS contamination of herbal extracts described in Figure 4 were confirmed by LAL assays (data not shown).

Although there is currently few publications discussing the LPS contamination problems in TCMH, it is worth-mentioning that LPS contamination may occur during processing, extraction and storage of TCMH. Therefore, inspection of LPS contamination in TCMH is necessary. Indeed, among our random surveys on the 35 herbal extracts derived from raw materials, concentrated herbal extracts and partially purified fractions, about 20% of them showed statistically significant LPS contamination above the concentration able to induce pro-inflammatory cytokine production. LPS contamination in these herbal extracts was confirmed by LAL assays. These results indicate that LPS contamination in TCMH is an unnegligible issue.

LPS structure contains both hydrophobic lipid A and polysaccharide region. LPS pharmacological antagonist polymyxin B, a cationic cyclic decapeptide isolated from *Bacillus polymyxa*, has been widely demonstrated to specifically neutralize the effect of LPS and exclude the possible

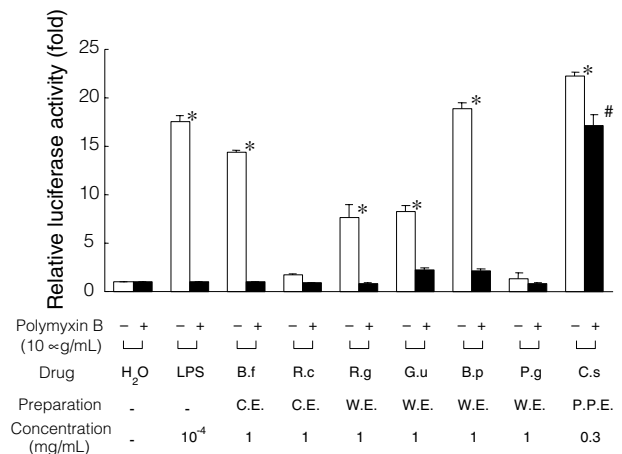


Figure 4. Detection of LPS contamination in various types of herbal extracts. The RAW 264.7/Luc cells were treated with LPS or herbal extracts for 5 hr, in the presence or absence of polymyxin B. Data shown are the mean and SEM of three independent experiments, and are presented as fold increase over control cells (vehicle only). “*” represents $p < 0.05$ as compared with control (vehicle). “#” indicates $p < 0.05$ as compared with control (vehicle plus polymyxin B). B.f.: *Bupleurum falcatum*; R.c.: *Rubus chingii*; R.g.: *Rehmannia glutinosa*; G.u.: *Glycyrrhiza uralensis*; B.p.: *Broussonetia papyrifera*; P.g.: *Panax ginseng*; C.s.: *Cordyceps sinensis*. C.E.: concentrated extract; W.E.: water extract; P.P.E.: partially purified fraction.

LPS contamination in the samples^(6,7,13,14). The neutralizing effect results from direct binding of the polycationic region of polymyxin B to the anionic lipid A portion of LPS⁽¹²⁾. It is possible that some components in TCMH may possess LPS-related structures and react with polymyxin B, leading to false negative results in our assays. However, the specificity problem may occur for all types of LPS detection methods. Other LPS inhibitors, such as anti-LPS antibody, can be added in our assays to further exclude this possibility⁽¹⁵⁾. On the other hand, there might be microbial LPS-related factors contaminated in TCMH, which cannot be neutralized by polymyxin B, thus giving false positive scores in our assays. For example, bacterial LPS-associated lipoproteins are possible candidates^(16,17). The activity of these lipoproteins was not affected by adding polymyxin B directly to the culture medium. However, activity of these lipoproteins can be removed by passing samples through polymyxin B agarose column or by prolonged heating⁽⁷⁾.

The most common method to measure LPS in a regular laboratory is end-point chromogenic LAL assay. Here we present an alternative method to measure LPS contamination. Comparison between our reporter-based assays and classical chromogenic LAL assays is described in Table 1. The reporter-based assay is less sensitive and quantitative than LAL assays. However, the reporter-based assay is less labor-intensive, with potential application for high-throughput LPS detection. In addition, the reporter assays directly provides information regarding NF- κ B bioactivity. Therefore, our reporter assay will provide a good comple-

ment for LAL assay, and can be generally applied to rapidly monitor the LPS contamination in TCMH. Chemical analysis by HPLC or GC-MS has also been reported to measure LPS contamination^(18,19). The LPS constituents, such as 3-hydroxy-lauric acid or 3-hydroxy fatty acids, served as marker substances for these methods. Chemical methods are quantitative but less sensitive in certain circumstances^(19,20). Furthermore, LPS derivatives detected by these methods cannot directly reflect the potential LPS-related bioactivity in the samples. Considering the highly complicated constituents of most crude herbal extracts, detection of LPS contamination in TCMH using chemical analysis may be more technically demanding.

ACKNOWLEDGMENTS

We would like to thank Dr. Tsun-Hsien Chuang in the Scripps Research Institute (La Jolla, USA) for providing plasmid pELAM1-Luc.

REFERENCES

1. Bone, R. C. 1991. The pathogenesis of sepsis. *Annu. Intern. Med.* 115: 457-469.
2. Salomao, R., Rigato, O., Pignatari, A. C., Freudenberg, M. A. and Galanos, C. 1999. Bloodstream infections: epidemiology, pathophysiology and therapeutic perspectives. *Infection* 27: 1-11.

Table 1. Comparisons for reporter-based assays and classical LAL assays

	Reporter-based assays	Chromogenic Limulus ameobocyte lysate (LAL) assays ^a
Detection targets	Reporter genes	Proenzyme activated by endotoxin
Time	A few hours	A few hours
Cost	Medium	High
Instrument	Luminometer	Spectrophotometer
Application	Qualitatively	Quantitatively
Detection limit ^b	1 ng/mL ^c (> 0.5 EU/mL)	0.01~0.1 EU/mL ^d
Advantages	Sensitive Low-background Large scale screening is feasible Convenient and robotic Directly measure NF- κ B-mediated bioactivity by LPS	Sensitive Can be quantitative
Disadvantages	Need to create a LPS-responsive stable cell line with integrated reporter plasmids Less quantitative and sensitive Need to add LPS-specific inhibitors to confer specificity	Samples possessing strong color may interfere with spectrophotometric absorbance reading Solution of pH beyond 7~8, strong ionic strength or high osmolarity may interfere with the results Multiple dilutions might be tested to find an appropriate dilution within narrow linear range (0.1~1 EU/mL or 0.01~0.1 EU/mL). Certain substances might affect results such as polynucleotides, solutions containing heavy metals, or surfactants

^aChromogenic limulus ameobocyte lysate assay QCL-1000 (Bio Whittaker, Walkersville, USA).

^bDetection limits may vary dependent on types and lots of LPS; different LAL kits may exhibit variable results.

^cThe EU is calculated based on product information provided by Sigma Co.

^dThe sensitivity of assays depends on the reaction conditions of the assays.

3. Ulevitch, R. J. and Tobias, P. S. 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu. Rev. Immunol.* 13: 437-457.
4. Beutler, B. 2000. Tlr4: central component of the sole mammalian LPS sensor. *Curr. Opin. Immunol.* 12: 20-26.
5. Anderson, K. V. 2000. Toll signaling pathways in the innate immune response. *Curr. Opin. Immunol.* 12: 13-19.
6. Gao, B., and 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.
7. Gao, B. and Tsan, M. F. 2003. Recombinant human heat shock protein 60 does not induce the release of tumor necrosis factor alpha from murine macrophages. *J. Biol. Chem.* 278: 22523-22529.
8. Medzhitov, R., Preston-Hurlburt, P. and Janeway, C. A. Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388: 394-397.
9. Byrd-Leifer, C. A., Block, E. F., Takeda, K., Akira S., and Ding A. 2001. The role of MyD88 and TLR4 in the LPS-mimetic activity of Taxol. *Eur. J. Immunol.* 31: 2448-2457.
10. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K. and Kimoto, M. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med.* 189: 1777-1782.
11. Morrison, D. C. and Jacobs, D. M. 1976. Inhibition of lipopolysaccharide-initiated activation of serum complement by polymyxin B. *Infect. Immun.* 13: 298-301.
12. Morrison, D. C. and Jacobs, D. M. 1976. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry* 13: 813-818.
13. Okamoto, M., Oshikawa, T., Tano, T., Ohe, G., Furuichi, S., Nishikawa, H., Ahmed, S. U., Akashi, S., Miyake, K., Takeuchi, O., Akira, S., Moriya, Y., Matsubara, S., Ryoma, Y., Saito, M. and Sato, M. 2003. Involvement of Toll-like receptor 4 signaling in interferon-gamma production and antitumor effect by streptococcal agent OK-432. *J. Natl. Cancer Inst.* 95: 316-326.
14. Teramoto, K., Nakamoto, Y., Kunitomo, T., Shoji, H., Tani, T., Hanazawa, K. and Kodama, M. 2002. Removal of endotoxin in blood by polymyxin B immobilized polystyrene-derivative fiber. *Ther. Apher.* 6: 103-108.
15. Kellogg, T. A., Lazaron, V., Wasiluk, K. R. and Dunn, D. L. 2001. Binding specificity of polymyxin B, BPI, LALF, and anti-deep core/lipid a monoclonal antibody to lipopolysaccharide partial structures. *Shock* 15: 124-129.
16. Rawadi, G. and Roman-Roman, S. 1996. Mycoplasma membrane lipoproteins induced proinflammatory cytokines by a mechanism distinct from that of lipopolysaccharide. *Infect. Immun.* 64: 637-643.
17. Rawadi, G., Roman-Roman, S., Castedo, M., Dutilleul, V., Susin, S., Marchetti, P., Geuskens, M. and Kroemer, G. 1996. Effects of *Mycoplasma fermentans* on the myelomonocytic lineage. Different molecular entities with cytokine-inducing and cytotoxic potential. *J. Immunol.* 156: 670-678.
18. Lyngby, J., H. Olsen, L., Eidem, T., Lundanes, E. and Jantzen, E. 2002. Quantification of lipopolysaccharides in outer membrane vesicle vaccines against meningococcal disease. High-performance liquid chromatographic determination of the constituent 3-hydroxy-lauric acid. *Biologicals* 30: 7-13.
19. Saraf, A., Larsson, L., Burge, H. and Milton, D. 1997. Quantification of ergosterol and 3-hydroxy fatty acids in settled house dust by gas chromatography-mass spectrometry: comparison with fungal culture and determination of endotoxin by a *Limulus* amoebocyte lysate assay. *Appl. Environ. Microbiol.* 63: 2554-2559.
20. Szponar, B., Norin, E., Midtvedt, T. and Larsson, L. 2002. Limitations in the use of 3-hydroxy fatty acid analysis to determine endotoxin in mammalian samples. *J. Microbiol. Method.* 50: 283-289.