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ORIGINAL ARTICLE

Protective Effect of Ethanol Extract of *Cuscuta chinensis* on Lipopolysaccharide-induced Acute Kidney Injury via Suppressing the Toll-like Receptors 4-nuclear factor-κB Pathway

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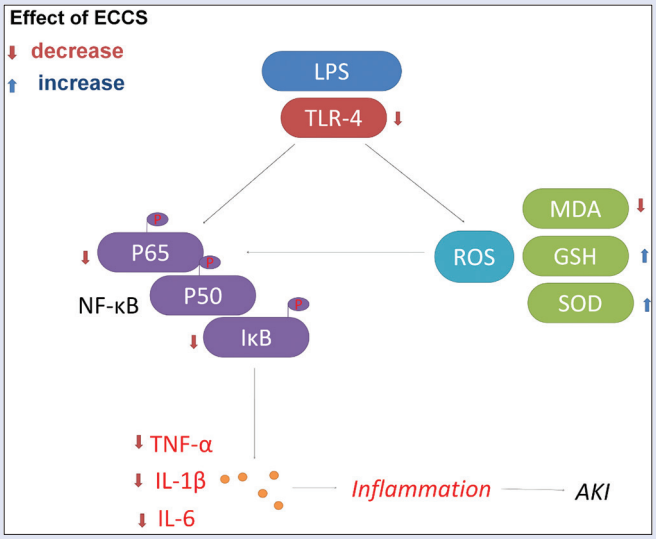
ABSTRACT

Background: The seed of *Cuscuta chinensis* Lam. (*Cuscuta, Convolvulaceae*), is widely used in traditional Chinese medicine, with a variety of biological activity. However, the efficacy of *C. chinensis* has not been investigated in renal injury caused by sepsis. **Objective:** Our research was carried out to evaluate the effect of ethanol extract of *C. chinensis* seeds (ECCS) on lipopolysaccharide (LPS)-induced acute kidney injury (AKI) in Kunming mice, and explore its underlying mechanisms. **Materials and Methods:** ECCS was administered orally (10, 30, and 100 mg/kg bodyweight, once daily) to the AKI mice for 14 consecutive days. On the 14th day, LPS (7 mg/kg) was administered to induce AKI. Blood urea nitrogen (BUN), creatinine (Cr), cytokines, and antioxidant index were estimated. The protein phosphorylation was tested by Western blot. **Results:** Results indicated that ECCS (100 mg/kg) decreased LPS-induced augmented BUN by 58.54% ($P < 0.001$) and reduced Cr by 61.57% ($P < 0.01$), respectively. In addition, the evident reversion of renal pathological damage was observed in ECCS-pretreated mice. ECCS (100 mg/kg) also exhibited a reduction of cytokines (tumor necrosis factor-α, interleukin [IL]-1 β, and IL-6, $P < 0.05$ for all) in AKI mice. Furthermore, ECCS blocked the activation of the nuclear factor-κB (NF-κB) (p65 and inhibitor kappa B subunit) and reduced the toll-like receptors 4 (TLR4) expression. **Conclusions:** The protective effect of ECCS against LPS-induced AKI was at least partially associated with suppressing of a TLR4-NF-κB signaling pathway, which provides evidence of the renal protective function of *C. chinensis* extract.

Key words: *Cuscuta chinensis*, inhibitor kappa B, p65, sepsis, toll-like receptor 4

SUMMARY

- The purpose of this study was to evaluate the effect of ethanol extract of *C. chinensis* seeds (ECCS) on lipopolysaccharide (LPS) induced acute kidney injury (AKI) in mice, and to explore its underlying mechanisms. The results showed the protective effect of ECCS against LPS induced AKI was at least partially associated with suppressing of TLR4-NF-κB signaling pathway.



Abbreviations used: AKI: Acute kidney injury; LPS: Lipopolysaccharide; NF κB: Nuclear factor κB; IκB: Inhibitor kappa B; IL: Interleukin; ECCS: Ethanol extract of C. chinensis seed; SOD: Superoxide dismutase; TLR4: Toll like receptor 4; BUN: Blood urea nitrogen; Cr: Creatinine; MDA: Malondialdehyde; PVDF: Polyvinylidene fluoride; TBST: TBS-Tween solution; iNOS: Inducible NOS; ROS: Reactive oxygen species; TH: T Helper; TBHB: Tert-butyl hydroperoxide; IFN-γ: Interferon-γ.

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INTRODUCTION

Acute kidney injury (AKI), characterized by abrupt loss of kidney function with tubular damage, is a serious and frequent complication of sepsis with high morbidity and mortality rate.[1] Lipopolysaccharide (LPS), known as lipoglycans and endotoxin, is commonly used to establish an experimental sepsis-induced AKI model.[2-4]

Mounting evidence indicates that nuclear factor-κB (NF-κB) activation and the subsequent coordinated expression of gene products may play crucial roles in the pathogenesis of AKI.[2,5] The majority of bacteria

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can bind to the surface of the cell membrane receptors (such as toll-like receptors), thereby stimulating the NF-κB signaling pathway to alter gene expression.[6] As an early transcription factor, NF-κB activation does not require new translational proteins to be regulated. The inactive NF-κB binds to the unphosphorylated inhibitor kappa B (IκB), which is phosphorylated by protein kinase and then degrade free NF-κB. When the inflammatory response occurs, the innate immune response cells are capable of releasing inflammatory factors. The transcription of the cytokines (interleukin [IL]-1, IL-6, IL-8), tumor necrosis factor-α (TNF-α), and COX-2 depends on the NF-κB activation.[7] Thus, agents that inhibit the production of inflammatory cytokines or NF-κB signaling pathways might prevent or attenuate tissue injury induced by LPS.

The seed of *Cuscuta chinensis* Lam. (*Cuscuta, Convolvulaceae*) have long been used in treatments for several diseases, including abortion, chyluria, glomerulonephritis, herpes zoster, as well as vitiligo.[8] Recent evidence indicates that *C. chinensis* shows various pharmacological effects including liver protection, kidney protection, antioxidant, antiaging, and antidepressant effects.[9,10] Most importantly, *C. chinensis* seed possesses strong anti-inflammatory effects, and the underlying mechanism was related to its upregulations of antioxidant enzymes and downregulations of pro-inflammatory cytokines, such as TNF-α and IL-1 β.[11] Moreover, the ethanol extract of *C. chinensis* seed (ECCS) could increase the activity of superoxide dismutase (SOD), glutathione-S-transferase, and glutathione reductase (GR), thereby inhibited liver damage caused by APAP.[9]

Although the ethanol ECCSs has shown anti-inflammatory benefits, its potential effects to protect against LPS-induced AKI still remained unclear. Therefore, to explore possible activities of ECCS for further applications, we established this study to investigate the effect of ECCS on early inflammatory response during LPS-induced AKI, with emphasizing its association with upregulation of toll-like receptor 4 (TLR4), NF-κB, and pro-inflammatory cytokines.

MATERIALS AND METHODS

Drugs and reagents

LPS (*Escherichia coli* 055:B5, 100 mg; item number: L2880) was from Sigma-Aldrich (Shanghai, China). Sodium chloride injection (Batch number: 1305080213) was obtained from the State Pharmaceutical Co., Ltd., Jilin Province, CN. Formaldehyde solution AR (40%, Batch number: 20110220) was from Longhai Reagent Factory, Dandong City, CN. Mouse blood urea nitrogen (BUN), creatinine (Cr), IL-10, IL-6, IL-1 β, malondialdehyde (MDA), SOD, and glutathione (GSH) ELISA kits were from Nanjing Jiancheng Institute (Jiangsu). Rabbit monoclonal antibodies IκBα, p65, p-p65, iNOS, and mouse monoclonal antibodies p-IκBα were obtained from Santa Cruz. Mouse TLR4 was from Abcam, Inc. (Cambridge, MA, USA).

Plant material and extract preparation

C. chinensis (Hebei Kang Pai Chinese Medical Materials Co., LTD, Inner Mongolia, Batch number: B13100102) was identified by Prof. Lu Jincai from Shenyang Pharmaceutical University (voucher specimen number: *Cuscuta*-20160723-002). *Cuscuta* sample powder was soaked with 75% ethanol by the ratio of liquid and material (1:10) for 12 h. Using a flash type extractor to extract the sample with an intermittent manner and revolving speed of 2800 r/min. This extraction was conducted three times at room temperature followed by filtration on Buchner funnel to give a clear filtrate. Combined filtrates were concentrated under reduced pressure and placed in a vacuum drying oven. The ethanol extract powder of *Cuscuta* was kept under 4°C. The yield of ethanol extract was approximately 24.5%. The *C. chinensis* was washed with water; vacuum dried in vacuum oven for 12 h, crushed, and went through 60 mesh sieves before stored in glass desiccators.

Animals

Experiments were performed on 18–25 g, 6–8 weeks old, male Kunming mice (SCXK 2010-0001). Mice were kept in a vivarium, maintained at 22°C ± 0.5°C in a 12 h alternating light-dark cycle (light on at 6:30 am) with free access to food and water.

Experimental designs

Mice were randomly split into five groups (n = 12): saline group, LPS group, and ECCS (10, 30 and 100 mg/kg) groups. ECCS groups were given ECCS (10, 30, and 100 mg/kg, i. g.) while saline group and LPS group were given saline (0.1 mL/10 g, i. g.) for 14 consecutive days. Two hours after the last administration, mice from ECCS groups and LPS group were administrated with a single injection of LPS (7 mg/kg, tail intravenous injection). Drug administration time: 8:00 am daily, the animals could acquire food and water freely. On the 14th day, 12 h after LPS injection, the mice were sacrificed. The serum was obtained for BUN and Cr detection. Later, the right kidney was harvested and then conserved in 4% paraformaldehyde for the renal histopathology measurement. At the same time, the left kidney was taken, placed in precold PBS immediately, then homogenized, which was then centrifuged (4°C, 3000 rpm, and 15 min). The supernatant was used for further analysis of antioxidant index (SOD, MDA, and GSH) and inflammatory cytokines, as well as for Western blot assay of TNF-α, iNOS, TLR4, NF-κBp65, and IκB.

Blood urea nitrogen and creatinine detection

Serum BUN and Cr levels were tested with a colorimetric detection kit using biochemical analyzer (Roche MODULE P800) following the manufacturer's protocol.

Detection of renal histopathology

To characterize the histological changes, the renal tissues were excised and fixed in 10% buffered formalin. The renal tissues were dehydrated in graded ethanol, embedded in paraffin, and the sections stained with hematoxylin and eosins were examined by light microscopy (OLYMPUS IX71 microscope, OLYMPUS DP72 Microscope refrigeration device with a color digital camera).

Cytokine assays

The renal sample was centrifuged at 3000 rpm, for 15 min at 4°C. The cytokine (IL-1 β, IL-6, and IL-10) concentrations in the supernatant were determined by ELISA according to the manufacturer's instructions.

Determination of malondialdehyde, superoxide dismutase, glutathione content

The kidney tissues were homogenized in saline and centrifuged (3000 g for 15 min at 4°C). MDA, SOD and GSH in were measured using commercially available kits.

Western blot analysis

The kidney tissue samples were dissected, weighed, and then homogenized on ice in homogenization buffer containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5 mM ethylenediaminetetraacetic acid, 0.1% SDS, and protease inhibitor mixture. Total protein was extracted from kidney tissue through grinder followed RIPI lysate (BI YUN TIAN Biotechnology Institute P0013B). Consequently, the protein concentration of the sample was determined by BCA Protein Assay Kit (BI YUN TIAN Biotechnology Institute P0012S) method. 10% SDS-PAGE electrophoresis was employed with a 40 μg sample volume and 70/120V constant voltage for 150 min, which followed by electrotransfer to a PVDF membrane

(0.45 μm). The membrane was blocked with 5% skim milk under 37°C for 1 h. After slightly washing, the membrane was incubated with murine internal reference protein β-actin antibody (1:500, Santa Cruz sc-47778), anti-TNF-α rabbit antibody (1:500, Santa Cruz sc-114), anti-p65 rabbit polyclonal antibody (1:100, Santa Cruz sc--109) and TLR4 polyclonal antibody (1:200, Bioworld Technology BS3489) in TBST plus 5% milk, followed by incubation with horseradish peroxidase-conjugated IgG (Zsbio, China) as the secondary antibody. The membrane was then washed with TBST buffer (3 × 10 min) before ECL developing reagent added. Finally, the membrane was scanned by UVP gel image analysis system, and the results were indicated by the ratio of the target protein bands' gray value and the internal reference protein β-actin bands' gray value.

Statistical analysis

Statistical analysis was carried out by SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) for Windows. All values were expressed as mean ± standard error of mean to evaluate the difference between groups, independent *t*-test, or one-way ANOVA followed by Tukey honest significant difference *post hoc* test was used. The level of significance was set at $P < 0.05$.

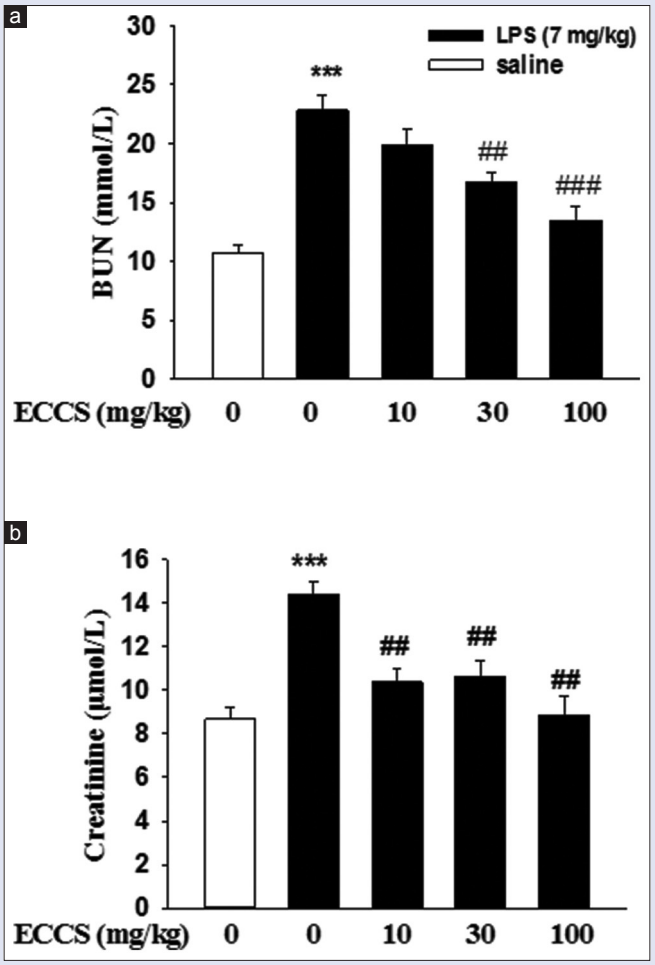

Figure 1: Effects of extract of *Cuscuta chinensis* seed on levels of blood urea nitrogen (a) and serum creatinine (b) of lipopolysaccharide-induced acute kidney injury mice. Data are shown as means ± standard error of mean (n = 10–12), $^{***}P < 0.001$compared to control group; $^{\#\#}P < 0.01$ versus lipopolysaccharide group; $^{\#\#\#}P < 0.001$ versus lipopolysaccharide group

RESULTS

Extract of *Cuscuta chinensis* seeds impact on serum blood urea nitrogen, creatinine levels

As shown in Figure 1, LPS induced significant increases of the levels of BUN and Cr in model group [$F_{(1,18)} = 9.07$, $P < 0.001$]. Compared with the model group, the application of *ECCS* produced a significant decrease of BUN and Cr in a dose-dependent manner (30 mg/kg: $P < 0.001$, 100 mg/kg: $P < 0.01$).

Pathology changes of kidney tissue

The pathological results showed that renal glomerular and tubular of the control group were normal under electron microscopy. As expected, LPS challenge caused tissue damage in the renal cortex and outer medulla [Figure 2], as showed by tubular epithelial cells sloughing, loss of brush border, tubular dilation, and distortion. In the ECCS-treated groups, the tubular damage was significantly ameliorated, especially the group of 100 mg/kg. The data confirmed that ECCS treatment markedly reduced tubular injury in LPS challenged mice.

Effect of extract of *Cuscuta chinensis* seeds on malondialdehyde, superoxide dismutase, and glutathione level

Treatment of mouse with a single dose of LPS (7 mg/kg) caused a significant increase in MDA levels as compared to the control group [Figure 3a, $F_{(1,19)} = 5.26$, $P < 0.01$], while SOD [Figure 3b, $F_{(1,18)} = 3.26$, $P < 0.05$] and GSH [Figure 3c, $P < 0.01$] markedly decreased. However, groups pretreated with ECCS (10, 30, and 100 mg/kg) showed significant declines in the MDA level ($P < 0.05$, for all). In addition, high-dose ECCS pretreatments (100 mg/kg) significantly boosted the SOD activities ($P < 0.05$) and increased the GSH level ($P < 0.05$), whereas 10 and 30 mg/kg ECCS showed no significant effect ($P > 0.05$).

Effect of extract of *Cuscuta chinensis* seeds on lipopolysaccharide-induced cytokine production

To evaluate the anti-inflammatory effects of ECCS in LPS-induced mouse AKI, the expressions of iNOS, TNF-α, IL-1 β, IL-6, and IL-10 were measured. IL-1 β, IL-6, and IL-10 concentrations in the serum were measured by Elisa, iNOS, and TNF-α by Western blot. The results showed that LPS caused a significant increase of the expression of iNOS, COX-2, TNF-α, and IL-6 compared with those in the control group ($P < 0.05$, for all). Pretreatment

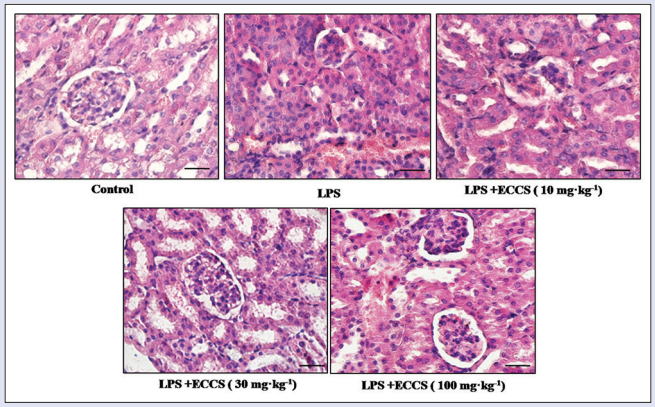

Figure 2: The effect of extract of *Cuscuta chinensis* seed on pathological changes (H and E, ×400) in kidney tissues of lipopolysaccharide (7 mg/kg)-induced acute kidney injury mice. Scale bar represents 20 μm

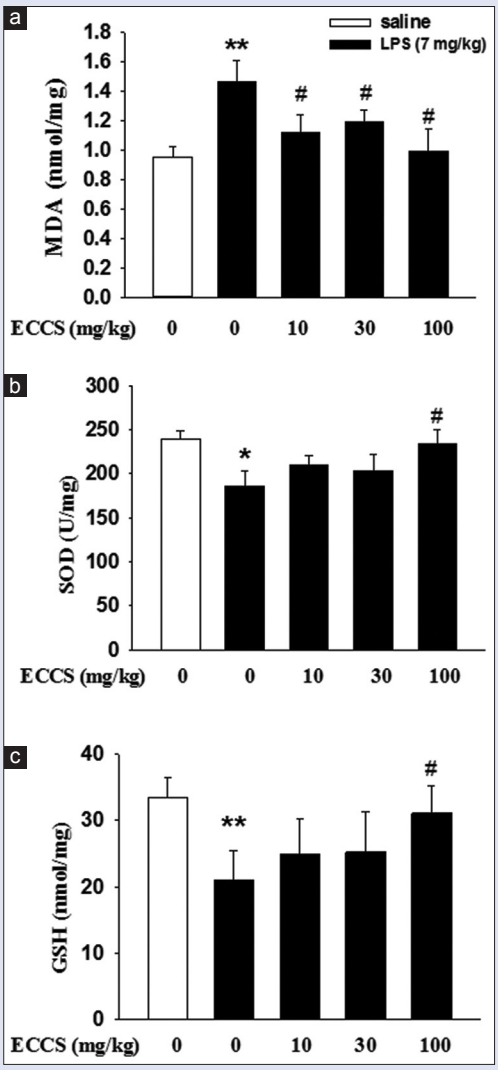

Figure 3: Effects of extract of *Cuscuta chinensis* seed on malondialdehyde, superoxide dismutase activity, and glutathione in kidney tissues of lipopolysaccharide-induced acute kidney injury mice. (a) renal tissue malondialdehyde level; (b) renal tissue superoxide dismutase activity; and (c) renal tissue glutathione content. Mice were injected with lipopolysaccharide with or without extract of *Cuscuta chinensis* seed (10, 30, 100 mg/kg) pretreatment. Data are presented as mean ± standard error of mean (n = 11–12). *P < 0.05, **P < 0.01 versus control group; #P < 0.05 versus lipopolysaccharide group

with ECCS (100 mg/kg) significantly increased the IL-10 level (P < 0.05), while decreased TNF-α, iNOS, IL-1 β, and IL-6 levels by 12.62%, 42.77%, 48.01%, and 52.24%, respectively [Figure 4]. ECCS (30 mg/kg) could also reduce the TNF-α level (P < 0.05), despite the fact that low dose of ECCS (10 mg/kg) did not show any effect.

Effect of extract of *Cuscuta chinensis* seeds on lipopolysaccharide-induced activation of nuclear factor-κB pathway

To probe the underlying mechanisms of ECCS, we examined the effect of ECCS on TLR4, NF-κBp65, and IκB expression in renal tissues. As shown in Figure 5, the TLR4/NF-κB signaling pathways were activated by LPS challenge (P < 0.05, for all). The expression of TLR4 of mice administrated at 100 mg/kg ECCS significantly decreased (P < 0.05). In ECCS (30 and 100 mg/kg) groups, the phosphorylation of P65 (p-P65/P65 ratio) and IκBα (p-IκBα/IκBα ratio) were suppressed in renal tissues (for all, P < 0.01). Unexpectedly, total P65 protein expression was increased by pretreatment of ECCS (100 mg/kg). These results showed that ECCS pretreatment, especially the high dose, efficiently downregulated of TLR4-dependent NF-κB signaling pathways in AKI mice induced by LPS.

DISCUSSION

In the present study, the anti-inflammatory effects of ECCS on LPS-stimulated AKI were evaluated in mice. The data showed that ECCS alleviated LPS-induced renal histological alterations, decreased serum BUN and Cr levels, inhibited pro-inflammatory cytokines productions, enhanced the antioxidation ability, and suppressed the NF-κB activations, as well as decreased expression of TLR4. These data demonstrated that ECCS possessed potential protective effects against LPS-induced AKI.

It is now well established that the chemical composition of *C. chinensis* seeds includes flavonoids, polysaccharides, alkaloids, steroids, volatile oils, lignans, and others. The main flavonoids including kempferol, quercetin, hyperoside, astragalin, and lignans play important roles in the pharmacological activity.[8,12,13] Lin *et al.* found that kempferol, the key active ingredient of flavonoids of *C. chinensis* methanol extract, could inhibit chemokines levels in LPS-stimulated dendritic cells *in vitro*.[14]

Oxidative stress is a crucial step in the development of LPS-induced kidney injury.[15] Nowadays, the level of ROS is known as a classical index of oxidative stress, which primarily attacks the polyunsaturated-fatty acids of cell and plasma membranes leading to the formation of MDA. The accumulation of MDA is commonly used as a marker to manifest the degree of lipid peroxidation, to some extent, the level of oxidative stress, and antioxidant status.[16] With the results of MDA assay, pretreatment with ECCS was found to have significant inhibitory effects on the formation of MDA, which clearly indicated that oxidative stress in kidney tissues of AKI mice was alleviated. In addition, antioxidants can abate the oxidative stress of AKI by direct elimination of free radicals or boosting defense systems of antioxidant enzymes. In this study, ECCS also markedly upregulated the activities of SOD and increased the GSH concentration. Combining with the results of MDA assay, we speculated that ECCS could effectively reduce oxidative stress in AKI. Similarly, former experimental studied had proved the hepatoprotective effect of ECCS against hepatic toxicity caused by acetaminophen through increased antioxidant capacity, while the aqueous extract was not found to affect SOD activity.[9] Interestingly, Another investigation that reported the aqueous extract could significantly inhibit the MDA production and ROS generation in MC3T3-E1 cell by TBHP-induced injury.[17] Despite little difference in the extraction process, these studies verify that the potential antioxidant activity of *C. chinensis*, which could be helpful in preventing oxidative stress in AKI.

There is increasing evidence that serum levels of TNF-α, IL-1 β, and IL-6 are correlated with the severity of endotoxemia and its consequence.[18,19] As expect, in the present study, pretreatment with ECCS significantly inhibited the production of TNF-α, IL-1 β, and IL-6. We found that ECCS was able to up-regulate IL-10, indicating that it may suppress host immune response and evade immune defenses. Furthermore, similar effects were found by Liao *et al.*, who indicated that the methanol extract of *Cuscuta* could significantly reduce carrageenan-induced edema paw by reducing the level of IL-1 β, IL-6, NF-κB, TNF-α, and COX-2. In another study, ethanol extract of *C. chinensis* exhibited potent antiaging effects on D-galactose-induced rat aging model.[20] Taken together, the inhibition of pro-inflammatory factors production was in accordance with the protective effects of ECCS against renal damage. Thus, the suppression of pro-inflammatory cytokines by ECCS treatment was supposed to contribute to its protective effects against AKI.

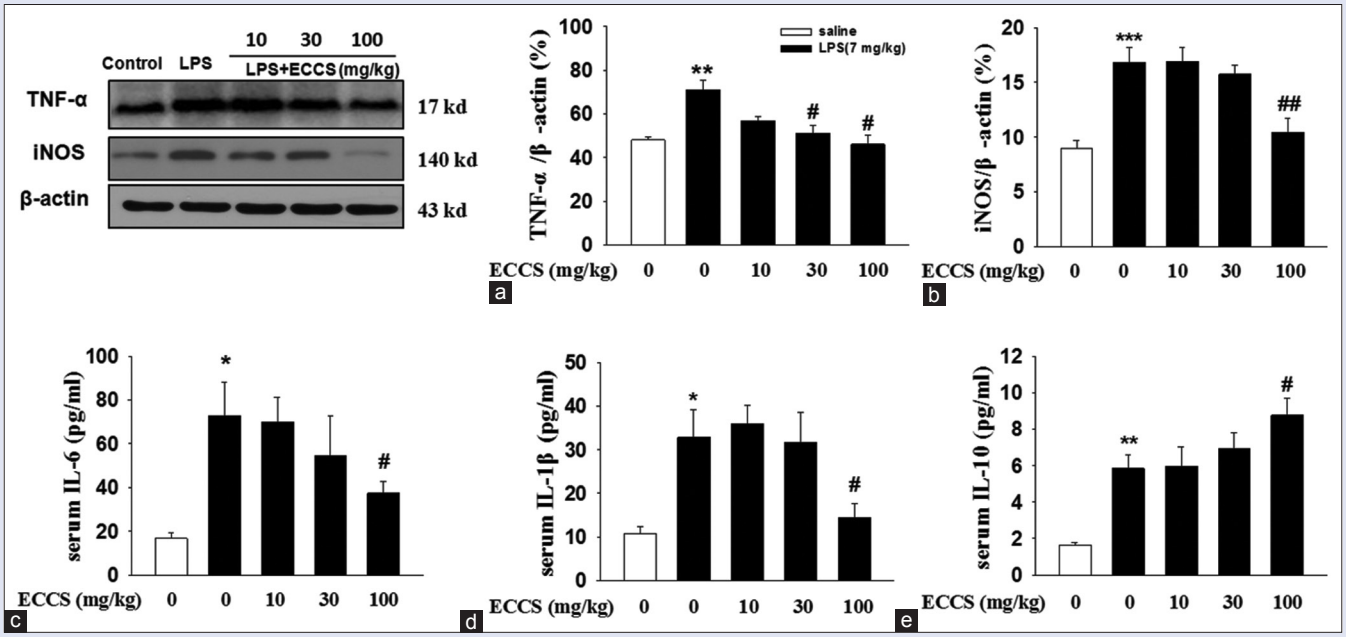

Figure 4: Effects of different concentrations of extract of *Cuscuta chinensis* seed on the levels of tumor necrosis factor-α (a), iNOS (b), IL-6 (c), IL-1β (d), and IL-10 (e) in lipopolysaccharide-induced acute kidney injury in mice. Kidney tissues and blood samples were collected for Western blot and ELISA determination. Data are presented as mean ± standard error of mean (n = 4-11). *P < 0.05, **P < 0.01, ***P < 0.001 versus control group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 versus lipopolysaccharide group. IL: Interleukin

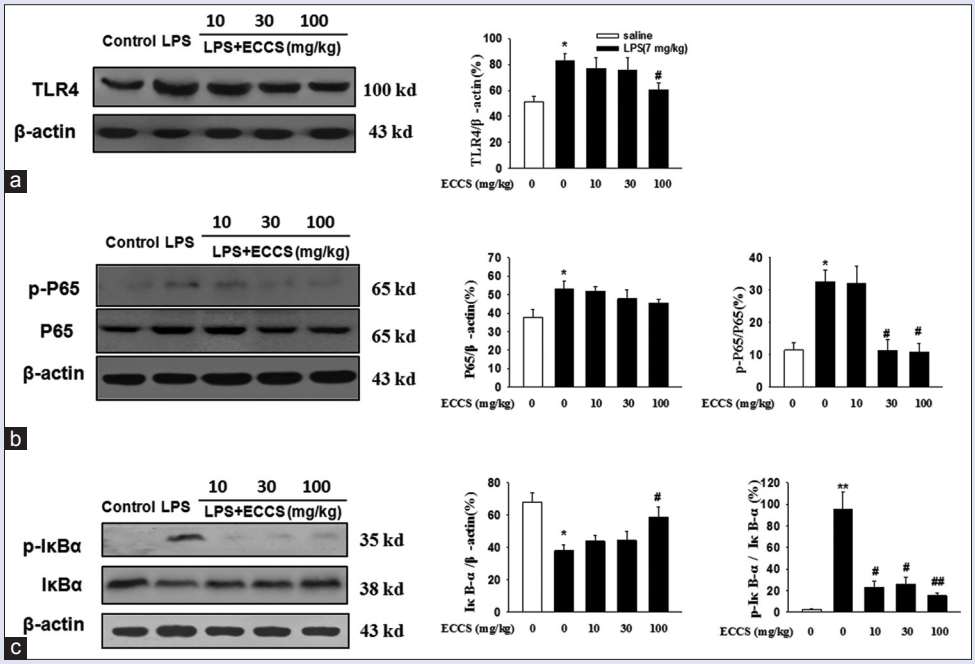

Figure 5: The effect of extract of *Cuscuta chinensis* seed on lipopolysaccharide-induced activation of the nuclear factor-κB signal pathway. (a) the protein expression of toll-like receptors 4; (b) total and phosphorylation level of nuclear factor-κB p65; (c) total and phosphorylation level of inhibitor kappa B alpha. The β-actin was used as the internal standard. The data shown here are from a representative experiment repeated three times with similar results. The relative optical densities normalized to β-actin are shown below the bands. Data are represented as mean ± standard error of mean (n = 4). *P < 0.05, **P < 0.01 versus control group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 versus lipopolysaccharide group

Studies in animal models and patients have shown that th2 cells cytokines, including IL-4, IL-6, and IL-10, which promote LPS-induced sepsis.[21] In addition, the previous studies have shown the sepsis immune environment from Th1 cells to Th2 reaction bias.[22] IL-4 is an important factor influencing the development of Th0 cells into Th2 cells. Elevated IL-4 is able to inhibit the activation of IL-12 and IFN-γ and subsequently promote the differentiation of juvenile Th0 cells into Th2 cells.[23,24] Herein, we present evidence showing that IL-4 and IL-10 were increased

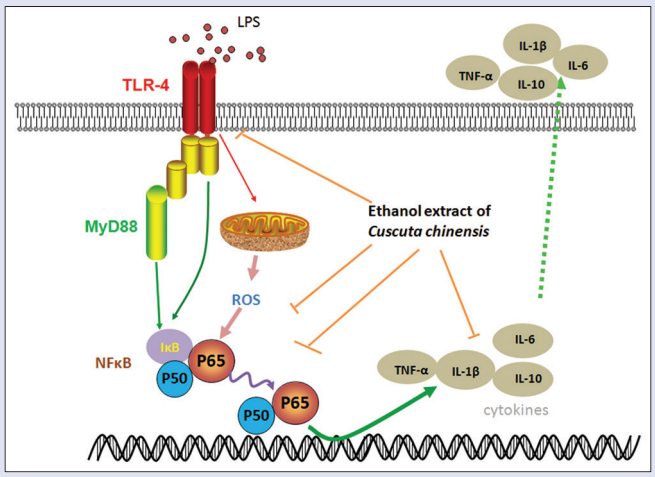

Figure 6: The possible underlying mechanism of extract of *Cuscuta chinensis* seed on the toll-like receptors 4-nuclear factor-κB signaling pathway. Extract of *Cuscuta chinensis* seed could inhibit the binding of lipopolysaccharide to toll-like receptors 4 in nuclear factor-κB signaling pathways, leading to reductions of pro-inflammatory cytokines productions, and attenuation of renal inflammatory responses

in LPS-treated mice, supporting the notion that LPS facilitating a Th2 response. Accordingly, our results suggest a possibility that ECCS could inhibit Th2 cell-mediated immune response in AKI.

It is noteworthy that are also important contributors to the inflammatory reaction of AKI.[25,26] As the major signaling receptor of LPS, TLR4 induces innate immune response, coupled with the regulation of genes encoding proinflammatory cytokines, thereby activating transcription factor as well as inducing intracellular signal transduction pathways. The canonical pathway of NF-κB cascade activated by LPS involves activation of TLR4, leading to recruitment of the adaptor molecules, such as IL-1R–associated kinase, activation of MAPKs, and IκB-kinase complex. Our study found that ECCS (100 mg/kg) pretreatment reversed LPS-induced remarkably increased phosphorylation of IκB and p65, by 87.75% and 66.36%, respectively. In addition, it was observed that the enhanced expression of TLR4 by LPS challenge were significantly decreased with pretreatments of ECCS at the doses of 30 and 100 mg/kg, which corresponded with the level changes of IκBα, p65, and other pro-inflammatory cytokine. In conclusion, these results suggested that ECCS might inhibit LPS-induced renal injury in mice, attributed to its roles in down-regulation of NF-κB pathways, as shown in Figure 6. It is noteworthy that because the extremely complicated NF-κB inflammatory pathways induced by LPS as well as the interaction between different signal pathways, the specific target of ECCS and its function are pending further study, especially the relationship with other upstream kinases of NF-κB and other signaling pathways.

CONCLUSIONS

This study demonstrated that ECCS could effectively attenuate the LPS-induced AKI in mice. The protective effects of CFE were associated with the modulations of TLR4-NF-κB signaling pathways. These experimental results suggested that ECCS was a potential therapeutic drug for AKI.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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