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Original Research Article

Protective effects of 4, 5-O-dicaffeoylquinic acid against mouse sepsis via down-regulation of TNF-α, IL-6 and IL-8

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Abstract

Purpose: *To investigate the protective effects of 4, 5-O-dicaffeoylquinic acid (DCQA) isolated from Xanthium sibiricum Patr. against mouse sepsis caused by cecal ligation/puncture (CLP) in vivo, as well as the molecular mechanisms of action involved.*
Methods: *DCQA (7.5, 15, and 30 mg/kg/day) were administered to the mice with sepsis and the survival rate was obtained. Tumor necrosis factor (TNF)-α and interleukin (IL)-6 were examined by enzyme-linked immunosorbent assay (ELISA). Subsequently, the lipopolysaccharide (LPS) neutralizing ability of DCQA (2, 4, and 8 μg/mL) was measured using limulus amebocyte lysate (LAL) test in vitro. Furthermore, the effect of DCQA (10, 20, and 40 μg/mL) on mRNA expression of TNF-α, IL-6, and IL-8 in LPS (100 ng/mL)-treated RAW 264.7 cells was assessed using quantitative real time-polymerase chain reaction (RT-qPCR) assay.*
Results: *DCQA significantly improved the survival rate of mice with sepsis caused by CLP (35, 50, and 65 %, respectively vs. 15 % for control, $p < 0.05$). LPS levels fell on co-incubation with DCQA in vitro. Moreover, ELISA and RT-qPCR results revealed that DCQA treatment lowered tendency in the mRNA expression of TNF-α, IL-6, and IL-8 ($p < 0.01$).*
Conclusion: *DCQA exhibits protective effects against sepsis in mice mediated by downregulating TNF-α, IL-6, and IL-8. Further studies, in animals and humans are requied to determine the safety and efficacy of DCQA in both animal and clinical management of sepsis.*

Keywords: *Xanthium sibiricum, 4,5-O-Dicaffeoylquinic acid, Sepsis, Cecal ligation and puncture, Lipopolysaccharide, TNF-α, IL-6, IL-8*

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INTRODUCTION

Sepsis is the common cause of death and a rapid-developing critical condition with a high mortality rate (> 20 %) in the intensive care units (ICU) of hospitals [1,2]. It is well-known that sepsis, generally caused by dysregulated inflammatory response to micro-organism infection, is characterized by life-threatening organ dysfunction and circulatory disturbance [3,4]. However, the related pathogenesis of sepsis is not completely clear yet, and the effective treatments are currently lacking. Therefore, it is necessary for us to develop an effective therapy to curing/attenuating sepsis.

Increasing evidence indicated that plant-derived extracts/compounds can be used as the potential resources for treatment of sepsis with few side-effects [5,6]. *Xanthium sibiricum* Patr., a specie of the family Asteraceae, is widely distributed in the Chinese provinces of Anhui, Shandong, Jiangxi, and Hubei [7,8]. It was reported that about 170 phytochemical compounds have been isolated and identified from this plant, including essential oil (sesquiterpene lactones and alkene), glycoside, phenol, alkaloid, and fatty acid [9,10]. The fruits of *X. sibiricum*, also called *Cangerzi* in Chinese, are commonly used for treating inflammatory diseases, such as acute and chronic rhinitis, itching diseases and urticaria [11,12].

As continuing efforts on separating constituents of *X. sibiricum,* 4, 5-O-dicaffeoylquinic acid (DCQA, Figure 1), a major phenylpropanoid derivative isolated from *X. sibiricum*, have been found to possess an anti-inflammatory property. In this study, the protective effects of DCQA against CLP surgery-induced sepsis in mice, the effect of DCQA on the viability of LPS-treated RAW 264.7 cells, as well as the underlying molecular mechanisms were examined.

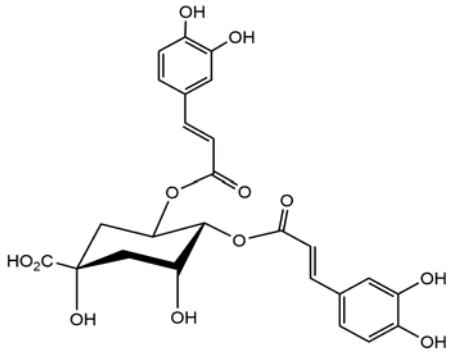

Figure 1: Chemical structure of 4, 5-O-dicaffeoylquinic acid (DCQA)

EXPERIMENTAL

Plant material

The fruits of *X. sibiricum* (voucher specimen no. 20170322343) were collected from Tong-Ren-tang (Beijing, China).

Animals

The animal experiments were carefully performed according to the Institutes of Health Guide for the Care of Animals [13], and were approved by the Committee for Animal Use of Wenzhou Central Hospital (approval no. 20170512ky-04). Male ICR mice (20 ± 2 g, 5-6 weeks) obtained from Shanghai laboratory animal center (Shanghai, China) mice, were kept in plastic pages (25 ± 2 °C; 55 ± 5 % humidity). They were adapted for 7 days with free diet and distilled water.

Chemicals and reagents

Lipopolysaccharide (LPS) was obtained from Sigma-Aldrich (Missouri, USA); ELISA kits for TNF-α and IL-6 from Beyotime Institute of Biotechnology (Haimen, China); quantitative RT-qPCR reaction kit and RNA Trizol reagents from Servicebio Company (Wuhan, China); silica-gel powder from Qingdao Marine Chemical (Qingdao, China).

Cell culture

RAW264.7 cells were collected from American Type Culture Collection (Virginia, USA) and cultured in the DMEM medium (5 % CO_2, 37 °C) with 10 % FBS and 1 % double resistance.

Identification of DCQA

X. sibiricum fruits (20 kg) were powdered through 80 meshes and extracted with petroleum ether to produce the petroleum ether fraction (FA). Then, the FA fraction was extracted with 75% ethyl alcohol by heating refluxing. After concentrating under a rotary evaporator, the residue (1.1 kg) was successfully suspended in water, which was then successively separated with different polar solvents (chloroform, ethyl acetate and n-butanol). Subsequently, the n-butanol fraction (FB, 200 g) was subjected to a chromatographic column (3 × 20 cm) over silica gel and then eluted with ethyl acetate-methyl alcohol (v/v, 30:1-25:1-20:1-15:1-10:1-5:1-1:1) to yield 7 fractions (Fraction 1-7). The fraction 2 was fractionated using Sephadex LH-20 gel column and C-18 reversed-phase silica gel column chromatography to yield the compound of DCQA. The identification of DCQA was determined by comparing NMR spectral data (Table 1) with previous reports [14].

Preparation of CLP model of mice

The CLP mice were established as previously described [3]. Briefly, after the mice were anesthetized with isoflurane (3 mL/kg), cecum was exposed by laparotomy under sterile condition. Then, the cecum was exteriorized and ligated with a silk suture, after which it was punctured with an 18-guage needle. Following with reposition of cecum, the abdominal incision was closed with double sutures.

Table 1: The ^{1}H-NMR and ^{13}C-NMR of DCQA

No.	δ_C	δ_H (J/Hz)	No.	δ_C	δ_H (J/Hz)
1	70.57		2’	116.12, 116.51	6.76 (s, J = 2.3,1H) 6.73 (s, J = 2.2,1H)
2	31.94	2.24 (d, J = 10.3, 1H) 2.36 (d, J = 10.4, 1H)	3’	146.38, 145.93	
3	63.76	4.03 (m, 1H)	4’	147.81, 148.02	
4	71.79	4.97 (dd, 1H)	5’	116.13, 116.21	6.97 (m, J = 8.3, 2H)
5	70.92	5.61 (br s, 1H)	6’	122.94, 123.03	6.82 (m, J = 8.3, J = 2.6, 2H)
6	37.42	2.21 (m, 1H) 2.49 (m, 1H)	7’	146.15, 145.92	7.52 (d, J = 16.7, 1H); 7.47 (d, J = 16.3, 1H)
7	171.95		8’	114.77, 114.63	6.34 (d, J = 16.5, 1H); 6.38 (d, J = 16.8, 1H)
1’	121.65, 121.77		9’	166.18, 166.24	

Eighty CLP mice were randomly divided into control group (saline, i.p.) and three DCQA treatment groups (7.5, 15, and 30 mg/kg, i.p.) (4 groups; n = 20). The survival percentages of mice were also observed within 96 h.

TNF-α and IL-6 levels in sepsis mice

The mice were sacrificed after CLP operation for 96 h, and 2 mL of blood sample was collected from each mouse. After centrifuging (10 min, 4 °C), the plasma was used for evaluation of TNF-α and IL-6 using ELISA kits.

Neutralizing effect of DCQA to LPS

To measure the neutralizing ability of DCQA to LPS *in vitro*, the LAL test was performed. Shortly, the DCQA at three concentrations of 2, 4, and 8 μg/mL were incubated with LPS of 1 ng/mL at 37 °C, and then the LAL reagent was supplemented to the mixture with equal amount. After mixing, the kinetic turbidity of the mixture was evaluated using the Tube Reader (Jinshan Biotech, Beijing, China).

Cell viability assay

RAW264.7 cells were seeded at 8 × 10^3 cells/well and then exposed to DCQA for 24 h at a series of concentrations in LPS presence of 100 ng/mL. Subsequently, MTT solution was supplemented to determine the cell viability of DCQA. The optical density was measured using Bio-Rad microplate reader (CA, USA) at 570 nm.

The mRNA expression of pro-inflammatory cytokines by RT-qPCR

Total RNA was extracted and transcribed into cDNA according to the introduction of RT-qPCR reaction kit. The primer sequences (Table 2) were synthesized by Shengong Biotech (Shanghai, China). The PCR reaction was conducted using an ABI StepOnePlus System (Biosystems, CA, USA), as 95 °C for 15 min, 95 °C for 30 sec and 50 °C for 60 sec (30 cycles) and 72 °C for 10 min.

Table 2: Primer sequences for RT-qPCR

Gene		Primer sequences
TNF-α	Forward primer	5′-CAGGTTCTGTCCCTTTCACTCACT-3′
	Reverse primer	5′-GTTCAGTAGACAGAAGAGCGTGGT-3′
IL-6	Forward primer	5′-TGGAGTACCATAGCTACCTGGAGT-3′
	Reverse primer	5′-TCCT-TAGCCACTCCTTCTGTGACT-3′
IL-8	Forward primer	5′-CTTTGTCCATTCCCACTTCTGA-3′
	Reverse primer	5′-TCCCTAACGGTTGCCTTTGTAT-3′
β-actin	Forward primer	5′-GGGAAATCGTGCGTGACATCAAAG-3′
	Reverse primer	5′-CATACCCAAGAAGGAAGGCTGGAA-3′

Statistical analysis

Data were presented as mean ± SD. The survival percentage was determined by Chi-squared of exact test. Other statistical differences were carried out using the two-tailed Student’s test by

SPSS software (Chicago, USA). $P < 0.05$ was regarded as statistically significance.

RESULTS

Effects of DCQA on survival percentage of CLP mice

After CLP operation, only three mice were survived for 96 h in the control group with a survival percentage of 15 % (Figure 2). However, the survived mice after CLP were dose-dependently increased by treatment with DCQA (7.5, 15, and 30 mg/kg), while the survival percentages were 35, 50, and 65 %. These findings suggested that treatment with DCQA markedly increased the survival percentage of CLP mice.

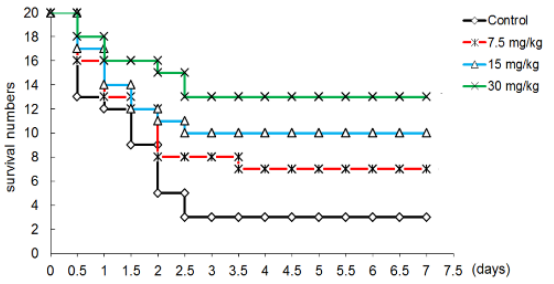

Figure 2: DCQA increased survival percentage of CLP mice. Data were expressed as mean ± SD (n = 20); *$P < 0.05$, compared to the control group

Effect of DCQA on TNF-α and IL-6 of CLP mice

As shown in Figure 3, after treatment with DCQA (7.5, 15, and 30 mg/kg), the TNF-α and IL-6 were all obviously decreased ($p < 0.01$), especially at 30 mg/kg.

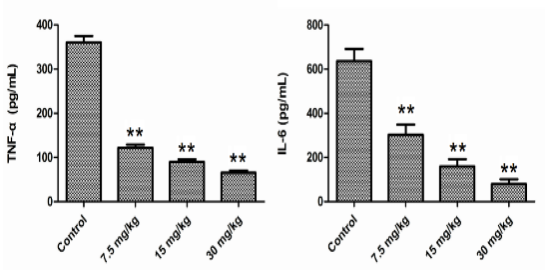

Figure 3: DCQA decreased TNF-α and IL-6 in CLP mice. Data were expressed as mean ± SD (n = 10). **$P < 0.01$, compared to the control group

DCQA decreased LPS activity i*n vitro*

As can be seen in Figure 4, LPS level was found to decrease following with co-incubation of DCQA (2, 4, and 8 µg/mL). These results indicated that DCQA has the capacity to neutralizing LPS level.

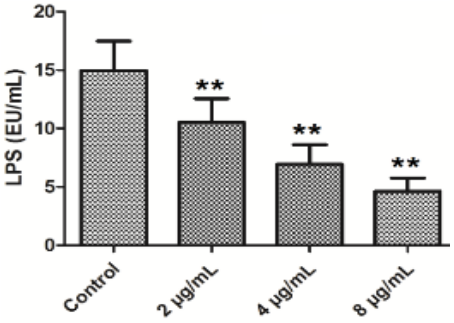

Figure 4: DCQA decreased LPS activity *in vitro*. Data were expressed as mean ± SD (n = 4); **$p < 0.01$, compared to the control group

Effect of DCQA on the viability of RAW 264.7 cells treated with LPS, and TNF-α, IL-6, and IL-8 mRNA expression

MTT assay revealed that DCQA at 5, 10, 20, 40, 80, and 160 µg/mL showed an extremely low cell proliferation inhibition on RAW 264.7 cells treated with LPS (Figure 5A). Furthermore, the RT-qPCR results (Figure 5B-D) displayed that the TNF-α, IL-6, and IL-8 were gradually decreased after incubation with DCQA (10, 20, and 40 µg/mL). These findings suggested that the protective effects of DCQA against sepsis mice may be associated with the anti-inflammatory property.

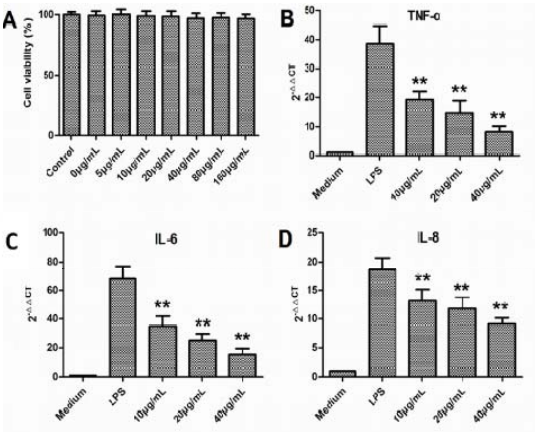

Figure 5: Effects of DCQA on cell viability, and mRNA expression of TNF-α, IL-6, and IL-8 levels in LPS-treated RAW 264.7 cells. Data were expressed as mean ± SD (n = 4); **$P < 0.01$, compared to the control group

DISCUSSION

Sepsis is a serious condition for which an effective drug or treatment strategy is needed.

There is no doubt that compounds and extracts derived from traditional Chinese herbal medicine are beneficial for human health. Increasing number of studies suggested that some natural products isolated from herbal medicine exhibited protective effects against sepsis [15]. This is the first investigation to study the protective effects of DCQA against sepsis by CLP-induced mice model and LPS-treated RAW 264.7 cells.

An inflammatory reaction may be not only related to the innate immune response, but can also be associated with the pathogenesis of several conditions such as sepsis and cancer [16]. Recent studies have confirmed that some pro-inflammatory cytokines of TNF-α and IL-6 act essential role in sepsis development. It has been indicated that the TNF-α is one of the important mediators in the produce of inflammatory response syndrome, and IL-6 and IL-8 are the downstream cytokines [17]. After the inflammatory response is activated, numerous secondary inflammation mesons can be triggered which may ultimately cause multiple organ dysfunction. An increasing number of investigations revealed that all three cytokines are related to the cytokine activation of cascade reaction in sepsis [18], and thus, suppression of the inflammatory response is critical in the treatment of sepsis. The results of this study indicated that DCQA can not only reduce TNF-α and IL-6 in sepsis mice induced by CLP surgery, but it can also inhibit the TNF-α, IL-6, and IL-8 expression in RAW 264.7 cells exposed to LPS.

Cecal ligation and puncture (CLP) is considered as a good experimental model to screen effective drugs for treating sepsis. It allows several intestinal bacteria to enter the abdominal cavity, followed by bacteria peritonitis and the transfer of bacteria into circulation [19]. The CLP surgery was applied to establish a pathological model of sepsis mice in the present study. On treatment with 7.5, 15, and 30 mg/kg of DCQA, the survival percentage of mice was increased to 35, 50, and 65 %, respectively. In addition, LPS is a trigger for sepsis and directly involved in the pathogenesis of a series of inflammatory response. Then, it activated immune reaction and damaged multiple organs [20]. RAW 264.7 cells treated with LPS were used for this investigation, and the TNF-α, IL-6, and IL-8 expression were decreased in all groups treated with DCQA (7.5, 15, and 30 mg/kg). Furthermore, the neutralization or inhibition of LPS would be beneficial in preventing severe sepsis, which can be viewed as a new therapeutic method [21]. The results in this study exhibited that DCQA had neutralizing effect on LPS *in vitro*.

CONCLUSION

The findings of this study, for the first time, show that 4, 5-O-dicaffeoylquinic acid (DCQA), a major phenylpropanoid derivative isolated from *X. sibiricum,* has the potential to be developed as an effective drug for the prevention of CLP-induced sepsis in mice. It acts by decreasing TNF-α, IL-6, and IL-8. This investigation provides a fresh insight into the medicinal potentials of the bioactive constituents of *X. sibiricum*.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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