**Esculin attenuates endotoxin shock induced by lipopolysaccharide in mouse and
NO production *in vitro* through inhibition of NF- κ B activation**

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1 **ABSTRACT**

2 Esculin, a coumarin compound derived from the traditional Chinese herbs such
3 as *Cortex Fraxini*, has long been used for treating inflammatory and vascular diseases.
4 In present study, we analyzed the role of esculin against macrophages and endotoxin
5 shock induced by lipopolysaccharide (LPS) in mice. Here, we demonstrated that
6 esculin suppressed inflammatory reactions in macrophages and protected mice from
7 LPS-induced endotoxin shock. We found that esculin significantly inhibited the
8 production of nitric oxide (NO) production via the inhibition of nuclear factor- κ B
9 (NF- κ B) activation in macrophages. In animal model, esculin pretreatment
10 significantly improved the survival rate of mice. LPS-induced increase of tumor
11 necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) in serum, lung, liver and
12 kidney were markedly inhibited by esculin. IL-10, an anti-inflammatory cytokine, was
13 up-regulated by esculin. Moreover, the histopathological analyses showed that esculin
14 significantly attenuated the tissues injury of lung, liver, kidney in endotoxic mice. In
15 addition, esculin significantly diminished the protein expression of NF- κ B p65 in lung,
16 liver, kidney, which resulted in lower levels of inflammatory mediators. These results
17 suggest that esculin may be a potential drug for treatment of various inflammatory
18 diseases.

19 **Keywords:**

20 Esculin; Endotoxin shock; Macrophages; Pro-inflammatory cytokines; Nuclear
21 factor-kappa B

1. Introduction

Inflammation is a process by which the body's chemicals and immune cells protect from infection with foreign materials, such as viruses and bacteria (Park et al., 2014). Among, sepsis is a severe systemic inflammatory disease with high mortality. It can be defined as a progressive failure of the circulation, characterized clinically by edema, shock, vascular leakage and organ failure (Li et al., 2014). Endotoxin has been adapted extensively to investigate the pathophysiological response to sepsis. Lipopolysaccharide (LPS), the cell wall component of gram-negative bacteria, is a common trigger of sepsis (Yan et al., 2013). The over-production of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6), involve in the tissue injuries and lethal shock (Li et al., 2014). Despite significant advances in antibiotic therapy and supportive care made in the past several decades, sepsis remains a severe disease and presents a high mortality rate in clinical. Therefore, it is urgently required to develop new therapies for sepsis.

During the inflammatory process, macrophages mediate the production of inflammatory mediators, such as TNF- α , IL-6, IL-10 and nitric oxide (NO) (Medzhitov, 2008; Freund et al., 2010; Smale, 2010). Due to many diseases are linked to the inflammatory response, substances including natural and synthetic chemicals that can mediate inflammatory process have been studied. Nuclear factor-kappa B (NF- κ B) is an important transcription factor that mediates gene expression for many chemokines, cytokines, growth factors and cell-adhesion molecules (Wong and Tergaonkar, 2009; Gasparini and Feldmann, 2012). Following activation by

inflammatory stimuli, such as LPS, cytokines, and viruses, the NF- κ B heterodimer is rapidly translocated to the nucleus, where it promotes the transcription of target genes, including TNF- α , IL-6 and NO (Park et al., 2014; Xie et al., 2014). Thus, NF- κ B pathway represents a valuable therapeutic target for preventing LPS-mediated inflammations.

As the important coumarin derivative, esculin (6,7-dihydroxycoumarin-6-o-glucoside, Fig.1) is the main bioactive constituents in *Cortex fraxini*. Esculin has various biological activities including anti-oxidant activity, intestinal anti-inflammatory activity, anti-cancer activity and growth inhibition of human leukemia cells (Li et al., 2012). Esculin inhibits lipid peroxidation and scavenges hydroxyl radicals in the rat liver (Kaneko et al., 2007) and exerts anti-inflammatory activity in both carrageenan- and zymosan-induced paw edema in mice (Stefanova et al., 1995). Martin et al. observed the gastroprotective effect of esculin in cold-restraint stress and pylorus ligation-induced ulcer models (Martin et al., 1991). Hence, as a potential candidate for anti-inflammatory therapy, we investigated the molecular mechanisms underlying the anti-inflammatory activities of esculin in activated macrophages and a murine model of endotoxin shock.

2. Materials and methods

2.1. Materials

Esculin was isolated from the stem bark of *Fraxinus chinensis Roxb.* Its chemical structure (shown in Fig.1) was confirmed by ^1H and ^{13}C nuclear magnetic resonance

(NMR) spectroscopy, and a purity greater than 98% was confirmed by liquid chromatography. As a positive control, dexamethasone (DEX), was purchased from Xi'an Lijun Pharmaceutical Company Limited (Xi'an, China). LPS (*Escherichia coli* serotype O55:B5) was supplied by Sigma (St. Louis, MO). The enzyme linked immunosorbent assay (ELISA) kits for mouse TNF- α , IL-6 and IL-10 were obtained from R&D Systems (Minneapolis, MN, USA). Griess reagent was provided by Sigma (St. Louis, MO, USA). The Protein Extraction Kit was purchased from Beyotime Institute of Biotechnology (Beijing, China). NF- κ B p65 polyclonal antibody and Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were provided by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyvinylidene fluoride membranes were supplied by Pall Gelman Laboratory (Ann Arbor, MI, USA). Other reagents were of commercially available analytical grade.

2.2. Animals

Healthy 6-7 weeks old male and female Kunming mice (20-25 g) were provided by the Experimental Animal Center, Xi'an Jiaotong University (Xi'an, China). Mice were raised under standard conditions with 12 h day/night cycles and allowed free access to food and water. All animal studies were carried out in accordance with the Care and Use of Laboratory Animals, published by the US National Institutes of Health. The mice were acclimatized to housing conditions for at least one week prior to experiments.

2.3. Survival Study

To evaluate the mortality rates, the esculin or DEX treatment groups were intragastric administrated with esculin (5, 10, 20 mg/kg) or DEX (5 mg/kg) for consecutive two days. One hour after the last administration, the mice except those in the control group received an intraperitoneal injection of LPS (30 mg/kg). The control group was given an equal volume of vehicle without LPS. The mortality of mice was recorded at 0, 12, 24, 36, 48, 60, and 72 h after LPS challenge in each treated group (n = 12/group).

2.4. Experimental protocol

The mice were randomly divided into six groups, and each group contains 12 mice: control group, LPS (30 mg/kg) group, esculin (5, 10, 20 mg/kg) plus LPS groups, and DEX (5 mg/kg) plus LPS group. The chosen doses and administration form of esculin and DEX were confirmed on the basis of our preliminary experiments. The control and LPS groups were pretreated with an equal volume of vehicle. The esculin or DEX treatment groups were intragastric administrated with different doses of esculin or DEX for consecutive two days. One hour after the last administration, the mice (except those in the control group) received an intraperitoneal injection of LPS (30 mg/kg). Six hours after LPS challenge, animals were killed by cervical dislocation after collecting blood through the retro-orbital plexus. Blood samples were separated by centrifuging for 10 min at $3000 \times g$ to obtain clear serum and frozen at $-20\text{ }^{\circ}\text{C}$ before biochemical estimation. After the mice were euthanized, the lung, liver and

kidney of mice were harvested immediately. Thereafter, each tissue was cut in half, and one half was immersed in 4% paraformaldehyde for histological observation, while the other half was stored at -80 °C until biochemical analyses.

2.5. Measurement of TNF- α , IL-6, IL-10 and NO production

The levels of TNF- α , IL-6 and IL-10 in serum were measured with the ELISA kits. The production of NO in serum were measured using Griess reagent. Tissue samples of lung, liver and kidney were placed in cold homogenization buffer at a ratio of 1 g/ml of saline, then homogenized and centrifuged at 2000 $\times$ g, 4 °C for 10 min. The levels of TNF- α , IL-6 and NO in the supernatant were detected using corresponding kits. All procedures were performed on the basis of the manufacturer's instructions.

2.6. Histopathological study

Tissues specimens of lung, liver and kidney were obtained for histopathological analysis at 6 h after LPS stimulation. Tissues were fixed in Bouin fluid for 48 h, dehydrated in graded alcohol and embedded in paraffin. Sections (5 μ m thick) were cut, mounted onto clear slides. After hematoxylin and eosin (H&E) staining, pathological changes of tissues were observed under a light microscope. The sections were assessed by an investigator who was blind to the arrangement.

2.7. Immunohistochemical study

At 6 h after LPS challenge, the lung, liver and kidney were collected for NF- κ B

p65 immunohistochemical study. The tissues of lung, liver and kidney were fixed in Bouin fluid, dehydrated in graded ethanol, embedded in paraffin, and sectioned (5 μ m thick). The tissue sections were dewaxed in xylene, rehydrated in graded alcohol, and immersed in citrate buffer (0.1 M, pH 6.0) in microwave oven for antigen retrieval. After cooling, the sections were washed with PBS and situated in 3% hydrogen peroxide at 37 °C for 10 min to wipe out the endogenous peroxidase. Afterwards, sections were incubated with goat serum at 37 °C for 20 min to reduce non-specific antibody-binding. After washing with PBS three times, the sections were incubated with the polyclonal antibody of NF- κ B p65 (diluted to 1:100) overnight at 4 °C. After being washed thrice in PBS, the sections were incubated with the biotinylated polyclonal rabbit anti-mouse second antibody (Dako, Carpinteria, CA, USA) for 30 min at room temperature. Subsequently, the sections were washed with PBS and stained with 3, 3'-diaminobenzidine (Sigma, St Louis, MO). Then the sections were stained with hematoxylin again and were dehydrated in graded ethanol. Finally, the sections were sealed in neutral gum. The immunohistochemical results of tissues were observed using a light microscope.

2.8. Cells

Macrophages were obtained according to the following procedure. Female Kunming mice were intraperitoneally injected with 1% sodium thioglycolate (1.5 ml/each) for seven consecutive days. Afterwards, the mice were killed by cervical dislocation, and the peritoneal macrophages were harvested by lavaging the peritoneal

cavity with 5 ml sterile PBS. Cells were collected by centrifugation at $1000 \times g$, 4°C for 10 min, and then washed three times with PBS. Cells were cultured in RPMI-1640 containing 10% fetal bovine serum at 37°C in 5% CO_2 .

2.9. Measurement of nitric oxide *in vitro*

In our previous study, the effect of esculin on macrophages viability were determined by MTT assay. The result indicated that cell viability was not significantly affected by up to 10^{-1} mg/ml of esculin ($>95\%$ cell viability). Therefore, subsequent *in vitro* assays were performed using LPS-stimulated macrophages at concentrations less than 10^{-1} mg/ml (Niu et al., 2015). Macrophages were seeded onto a 24-well plate at a density of 5×10^5 cell/ml. The cells were then treated with various doses of esculin (final concentrations of 10^{-3} to 10^{-1} mg/ml) for 24 h before LPS stimulation ($10 \mu\text{g/ml}$) for 12 h. The amount of nitric oxide released by the mouse macrophages was detected by Griess reagent.

2.10. Western blot analysis for NF- κ B p65 *in vivo* and *in vitro*

Six hours after LPS challenge, tissue samples were harvested and immediately frozen in liquid nitrogen until use. The macrophages (3×10^6 cells in 2 ml) seeded in 6-well plate were pretreated with esculin (final concentrations of 10^{-3} to 10^{-1} mg/ml) for 24 h before exposure to LPS ($10 \mu\text{g/ml}$) for 12 h. The extraction of proteins from tissues and cells were performed by a Protein Extraction Kit, and protein concentration were measured by BCA protein assay kit. Equal amounts of total

1 proteins from each group were loaded in each well and separated by 10% sodium
2 dodecyl sulfatepolyacrylamidgel. Subsequently, proteins were transferred onto
3 polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat
4 dry milk in TBST for 2 h at room temperature, and then incubated with the primary
5 antibodies (anti-NF- κ B p65, 1:500; anti-GAPDH, 1:20000) overnight at 4 °C. The
6 membranes were washed four times with TBST at room temperature and then
7 incubated with an anti-rabbit horseradish peroxidase-conjugated antibody (1:20000
8 dilutions in TBST) at 37 °C for 1 h. Immunoreactive proteins were detected by
9 enhanced chemiluminescence system (GE Healthcare, Little Chalfont,
10 Buckinghamshire, UK). The western blot analysis of GAPDH was performed as the
11 internal control of protein loading.

12

13 *2.11. Statistical analysis*

14 All data were presented as mean $\pm$ standard error of mean (S.E.M.). Differences
15 between mean values of normally distributed data were analyzed by one-way analysis
16 of variance (ANOVA) and two-tailed Student t-test. Statistical analyses and column
17 charts were finished by the GraphPad Prism software (California, USA). The
18 comparisons among survival rates of the groups were performed by a Kaplan-Meier
19 curve and log-rank test with SPSS software. Statistical significance was accepted at P
20 < 0.05 .

21

22 **3. Results**

3.1. Effects of esculin on LPS-induced mortality in mice

LPS-induced endotoxin shock represented a good model for exploring the inflammatory process *in vivo* (Remick and Ward, 2005). As shown in Fig.2, esculin notably reduced LPS-induced death in mice. The survival rates during 72 h in esculin (5, 10, 20 mg/kg) and DEX (5 mg/kg) treatment groups were 50%, 58.33%, 75%, and 83.33% respectively, which were significantly higher than that in LPS group ($P < 0.05$). Kaplan-Meier survival analysis showed that pretreatment with esculin (5, 10, 20 mg/kg) effectively reduced LPS-induced death.

3.2. Effects of esculin on the levels of TNF- α , IL-6, IL-10 and NO in serum

The levels of TNF- α , IL-6, IL-10 and NO in serum were measured with the corresponding kits according to the manufacturer's instructions. As shown in Fig.3, mice treated with LPS alone showed a marked increase in the levels of TNF- α , IL-6 and NO compared to the control group. Pretreatment with 5, 10, 20 mg/kg esculin notably attenuated LPS-induced up-regulation of TNF- α , IL-6 and NO levels in serum. DEX pretreatment also effectively inhibited these cytokines, compared to the LPS-alone. IL-10 is known as an anti-inflammatory cytokine to limit inflammatory responses, and the administration of esculin markedly enhanced IL-10 level compared with that in LPS group.

3.3. Effects of esculin on LPS-induced TNF- α and IL-6 production in lung, liver and kidney

As shown in Fig.4, stimulation with LPS markedly increased the expression of TNF- α and IL-6 in lung, liver and kidney compared to the control group ($P < 0.01$). Pretreatment with esculin significantly attenuated LPS-induced TNF- α and IL-6 levels in lung tissues (Fig.4A₁ and Fig.4B₁). LPS-induced TNF- α and IL-6 levels in liver were markedly lowered, when pretreated with 5, 10, 20 mg/kg esculin (Fig.4A₂ and Fig.4B₂). Moreover, esculin treatment effectively decreased pro-inflammatory cytokines TNF- α and IL-6 production in kidney tissues, compared to LPS group (Fig.4A₃ and Fig.4B₃). Additionally, DEX pretreatment could markedly reduce the levels of TNF- α and IL-6 in the lung, liver and kidney of mice. These results indicates that esculin play an important role in regulating pro-inflammatory cytokines levels of TNF- α and IL-6 in lung, liver and kidney tissues.

12

3.4. Effects of esculin on pathological injury of lung, liver and kidney

The histological examination showed no pathological abnormalities in the blank control mice (Fig.5A, A₁, A₂). In contrast, the mice expose to LPS showed significant inflammatory injuries characterized by pulmonary edema and interstitial cell infiltration in the lung (Fig.5B), hepatic cell necrosis and hemorrhage in the liver (Fig.5B₁), and tubular renal cell swelling and hemorrhage in the kidney (Fig.5B₂). However, pretreatment with esculin significantly ameliorated many of the symptoms of tissue pathology in the mice (Fig.5C-E, C₁-E₁, C₂-E₂). Meanwhile, DEX abolished the histopathological injuries in the lung, liver and kidney of LPS-challenged mice (Fig.5F, F₁, F₂). Meanwhile, the injury score of lung, liver and kidney (Fig.5G, G₁, G₂)

1 were significantly reduced in esculin treatment group, compared with the LPS-alone
2 group ($P < 0.05$).

3 4 3.5. Effects of esculin on NF- κ B p65 protein expression in LPS-induced endotoxin 5 shock model

6 The effects of esculin on NF- κ B p65 protein expression in mice were evaluated by
7 immunohistochemical study. In the control group (Fig.6A, A₁, A₂), the
8 immunolabelling of NF- κ B p65 in lung, liver and kidney were scarcely distributed.
9 Compared with the control group, intense staining of NF- κ B p65 in lung, liver and
10 kidney tissues were observed in LPS-challenged endotoxin shock mice (Fig.6B, B₁,
11 B₂). However, pretreatment with esculin (5, 10, 20 mg/kg) or DEX (5 mg/kg)
12 significantly inhibited the increase of NF- κ B p65 protein expression in lung, liver and
13 kidney tissues (Fig.6C-F, C₁-F₁, C₂-F₂). The results in Fig.6G were the mean density
14 for NF- κ B p65 protein expression in lung, liver and kidney. These results showed that
15 esculin could effectively inhibit the activation of NF- κ B in mouse model of endotoxin
16 shock.

17 18 3.6. Esculin inhibited NF- κ B activation in LPS-induced endotoxin shock model

19 To fully understand the protective effects of esculin on endotoxin shock mice, we
20 further investigated the protein expression of NF- κ B p65 in tissues by western blot
21 analysis. As shown in Fig.7, mice challenged with LPS showed obvious increase of
22 NF- κ B p65 in lung, liver and kidney tissues when compared with the control group.

1 However, esculin (5, 10, 20 mg/kg) or DEX (5 mg/kg) pretreatment significantly
2 inhibited the NF- κ B p65 protein expression in lung, liver and kidney. These results
3 showed that esculin effectively relieved LPS-induced endotoxin shock might be
4 relevant to the inhibition of NF- κ B activation.

6 3.7. *Effects of esculin on NO production in LPS-stimulated macrophages*

7 As shown in [Fig.8](#), LPS stimulation significantly increased the production of NO
8 compared to the control ($P < 0.01$). Pretreatment with esculin significantly reduced
9 LPS-induced NO production in macrophages ($P < 0.05$).

11 3.8. *Esculin inhibited LPS-induced NF- κ B activation in LPS-stimulated macrophages*

12 In present study, the effect of esculin on LPS-stimulated NF- κ B activation in
13 macrophages was evaluated. As shown in [Fig.9](#), macrophages challenged with LPS
14 showed obvious increase of NF- κ B p65 when compared with the control group ($P <$
15 0.001). However, esculin (10^{-3} , 10^{-2} , 10^{-1} mg/ml) pretreatment significantly inhibited
16 the LPS-induced NF- κ B p65 protein expression *in vitro* ($P < 0.05$). These results
17 showed that esculin significantly decreased LPS-induced NO production *in vitro*
18 might be relevant to the inhibition of NF- κ B activation.

20 4. Discussion

21 Sepsis is defined as the systemic inflammatory response to external stimulation
22 ([Melamed and Sorvillo, 2009](#)). The detrimental effect of LPS challenged mice is

1 usually considered as an experimental model for endotoxin shock or sepsis ([Remick](#)
2 [and Ward, 2005](#)). Herein, we demonstrated that esculin pretreatment provided
3 protection against LPS-induced endotoxin shock in mice. The present study showed
4 that esculin pretreatment significantly reduced LPS-induced mortality and attenuated
5 the tissue injuries. Meanwhile, esculin significantly decreased the increase of
6 pro-inflammatory cytokines and mediator in serum and tissues from lung, liver and
7 kidney. Moreover, protein expression of NF- κ B p65 in tissues were also notably
8 reduced by esculin pretreatment when compared with the LPS group. These results
9 suggest that esculin is a potential agent for endotoxin shock treatment.

10 LPS challenge can initiate acute and chronic inflammation in mice, even lead to
11 death. In present study, mice exposed to LPS exhibited a high mortality. However,
12 pretreatment with esculin significantly reduced the mortalities, suggesting that esculin
13 pretreatment effectively protected mice from LPS-induced death. Besides, the
14 morphological study of the normal tissues showed no damage in lung, liver and
15 kidney. However, in the group treated with LPS-alone, it was observed that there were
16 pulmonary edema and interstitial cell infiltration in the lung, hepatic cell necrosis and
17 hemorrhage in the liver, and tubular renal cell swelling and hemorrhage in the kidney.
18 Following esculin pretreatment, these pathological injuries were markedly attenuated.
19 These results further support the protective effects of esculin in treating endotoxin
20 shock induced by LPS.

21 LPS-induced endotoxin shock exhibits some relevant features of the clinical
22 disease such as interstitial edema, neutrophil accumulation, inflammatory cytokines

1 release, and injury ([Sato et al., 2002](#)). It is generally admitted that sepsis is caused by
2 excessive inflammatory response to invading pathogens. The downregulation of
3 pro-inflammatory cytokines (TNF- α and IL-6) and the upregulation of
4 anti-inflammatory cytokines (IL-10 and IL-13) have been shown to be the major
5 factor in treating sepsis. TNF- α and IL-6 contribute to the severity of lung injury
6 ([Giebelen et al., 2007](#)). TNF- α is a representative and pleiotropic pro-inflammatory
7 cytokine in the inflammatory reaction. It stimulates the production of COX-2 and
8 iNOS ([Goldring and Goldring, 2004](#)) and triggers inflammation, injury and
9 carcinogenesis in various tissues ([Faubion and Gores, 1999](#)). It has already been
10 demonstrated that the concentration of TNF- α enhanced in BALF from patients with
11 ALI ([Zhang et al., 2011](#)). Another significant pro-inflammatory cytokine is IL-6,
12 which is generated by different cells and possesses pleiotropic effects on various
13 tissues. IL-6 regulates genes expression involved in cell cycle progression and
14 suppression of apoptosis ([Lin and Karin, 2007](#)). According to the previous researches,
15 an increasing level of IL-6 was observed in inflammatory diseases such as fever,
16 sepsis, ulcerative colitis, rheumatoid arthritis and mesangial glomerulonephritis
17 ([Autschbach et al., 1998](#)). IL-10 is known as an anti-inflammatory cytokine, which
18 limit inflammatory responses and TNF- α circulation ([Oku et al., 2002](#)). Previous
19 studies have shown that IL-10 deficient mice or mice treated with a monoclonal
20 antibody against IL-10 showed higher plasma levels of pro-inflammatory cytokines
21 and increased mortality, whereas administration of recombinant IL-10 to
22 LPS-stimulated mice showed protective effects against pro-inflammatory cytokines

1 production and lethality ([Gérard et al., 1993](#)). The beneficial effects of IL-10 could be
2 due at least in part to the reduction of TNF- α release. IL-10 is expressed by many
3 immune cells, including dendritic cells (DCs), macrophages and neutrophils. The
4 activation of DCs and macrophages involves the recognition of pathogen-derived
5 products by patterns recognition, which triggers the expression of cytokines and
6 factors. Through secretion IL-10, macrophages down-regulate their own activation
7 and pro-inflammatory mediator secretion in an autocrine loop ([Fiorentino et al., 1991](#)).
8 IL-13, another important anti-inflammatory cytokine, is expressed by activated Th2
9 cells and mast cell. It has been reported that IL-13 suppresses IL-1 β and TNF- α
10 production from both human peripheral blood mononuclear cells and macrophages in
11 vitro ([Cosentino et al., 1995](#); [Deleuran et al., 1995](#)). The anti-inflammatory properties
12 make IL-13 a candidate for the treatment of macrophage-mediated inflammatory
13 conditions. In present study, we found that the levels of TNF- α and IL-6 in serum and
14 tissues were significantly increased in LPS-challenged mice compared with the
15 control group. Pretreatment with esculin effectively reduced TNF- α and IL-6 content
16 in serum and tissues from lung, liver and kidney. Meanwhile, pretreatment with
17 esculin increased the content of IL-10 in serum. Although the effects of esculin on
18 IL-13 levels wasn't detected in this study, on the basis of previous experiments we
19 hypothesized that esculin might be change the content of IL-13. These results
20 demonstrated the protective effect of esculin might be with related with the inhibition
21 of pro-inflammatory cytokines and induction of anti-inflammatory cytokines.

22 NO exerts various regulatory functions on inflammatory conditions and plays a

1 key role in the regulation of immune responses. During the course of inflammatory
2 response, plenty of NO formed by iNOS surpass the physiological amounts of NO
3 (Xie and Nathan, 1994; Liu et al., 2012), which is of interest to our study of the
4 release of pro-inflammatory cytokines TNF- α and IL-6. In our study, the production
5 of NO in serum was found to be notably reduced in esculin-treated mice compared to
6 those in LPS-alone group.

7 Toll-like receptor 4 (TLR4), which recognizes various pathogen-associated
8 molecular patterns derived from bacteria, plays a central role in sepsis induced by
9 endotoxin (Takeuchi et al., 2001). TLR4 has been extensively studied as a potential
10 drug target in cancer, rheumatoid arthritis, and allergic asthma. Once stimulated by
11 LPS, TLR4 can activate the downstream NF- κ B signaling pathway, subsequently the
12 release of inflammatory cytokines (Akira and Takeda, 2004). NF- κ B, the downstream
13 transcription factors of TLR4 signaling, promotes the expression of genes encoding
14 inflammation-mediated protein such as TNF- α , IL-6, COX-2 and iNOS
15 (Pålsson-McDermott and O'Neill, 2004; Kagan et al., 2008). Previous studies
16 demonstrated that increased activation of NF- κ B is found in peripheral blood
17 mononuclear cells, alveolar macrophages, and neutrophils from patients with sepsis. A
18 greater persistent nuclear accumulation of NF- κ B is associated with higher mortality
19 and more persistent pulmonary injury (Wu et al., 2007). In addition, it has been
20 proved that the expression of NF- κ B-dependent pro-inflammatory mediators was
21 diminished, and the severity of endotoxemia-induced ALI was decreased by inhibition
22 of nuclear translocation of NF- κ B (Moine et al., 2000). In present study, we observed
23 that LPS challenge caused a significant increase in the protein expression of NF- κ B
24 p65 in the lung, liver and kidney compared with the control mice, whereas
25 pretreatment with esculin notably reduced the protein expression of NF- κ B p65 in

1 tissues. These results indicated that the inhibition of NF- κ B activation might play an
2 important role in the process of antagonizing endotoxin shock by esculin. On the basis
3 of previous study, we hypothesized that suppression of NF- κ B activation by esculin
4 may be related with the interference of TLR4 pathway, however, the deeply
5 mechanism of esculin in sepsis needs to be studied in the future.

6 In experimental models, activation of macrophages could be mimicked by LPS
7 administration. LPS, a bacterial lipopolysaccharide, has the potency to activates
8 inflammatory responses in macrophages (Fujiwara and Kobayashi, 2005).
9 LPS-challenged macrophages produce inflammatory mediators such as NO, TNF- α
10 and IL-6, to regulate the pathogenesis of immune responses and inflammation
11 (Kaplanski et al., 2003; Fujiwara and Kobayashi, 2005; Li et al., 2009). To further
12 explore the effect of esculin on inflammatory mediator production, the *in vitro* model
13 of LPS-challenged macrophages was adopted to examined the effect of esculin on NO
14 production and clarified the possible mechanisms involved. We found that the
15 production of NO in serum was notably reduced in esculin-treated mice compared to
16 those in LPS group. The transcription factor NF- κ B has been shown to be a significant
17 regulator which can regulate functionally various pro-inflammatory mediators, such
18 as NO (Li et al., 2011). In present study, the effect of esculin on transcriptional
19 activations of NF- κ B was evaluated. Esculin significantly inhibited LPS-induced
20 protein expression of NF- κ B p65 in macrophages. Overall, the results showed that
21 esculin blocks the NF- κ B activation by reducing the protein expression of NF- κ B p65
22 and thereby inhibits the production of NO in macrophages.

23

5. Conclusion

In conclusion, the present study showed that esculin had the protective effect on LPS-induced endotoxin shock in mice. The protective effect of esculin seemed to result from its inhibition of NF- κ B activation, and subsequently caused a remarkable reduction in pro-inflammatory cytokine levels, and alleviating tissue injuries. Thus, the survival rate of mice was increased. *In vitro*, esculin inhibited LPS-induced NO production in macrophages at least partially through the blockade of NF- κ B pathway. These results implicates that is a potential therapeutic agent for the treatment of inflammatory diseases. Further researches are still needed to confirm the protective mechanism of esculin.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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1 **Figure legends**

2 **Fig.1.** Chemical structure of esculin.

3

4 **Fig.2.** Effects of esculin on LPS-induced mortality in mice (n = 12/group). The
5 survival was observed at 0, 12, 24, 36, 48, 60 and 72 h after LPS challenge and the
6 percent survival was expressed as Kaplan-Meier survival curves. ^{###}P < 0.001 when
7 compared with the control group; *P < 0.05 and ***P < 0.001 when compared with
8 the LPS group.

9

10 **Fig.3.** Effects of esculin on the levels of TNF- α (A), IL-6 (B), IL-10 (C) and NO (D)
11 in serum (n = 12/group). Blood samples were collected at 6 h following LPS
12 challenge. The levels of TNF- α , IL-6, IL-10 and NO in serum were measured with the
13 corresponding kits. Data presented are mean $\pm$ S.E.M. [#]P < 0.05, ^{##}P < 0.01 and ^{###}P <
14 0.001 versus the control group; *P < 0.05 and **P < 0.01 versus the LPS group.

15

16 **Fig.4.** Effects of esculin on TNF- α and IL-6 levels in lung, liver and kidney from
17 LPS-challenged endotoxin shock mice (n = 12/group). Six hours after LPS challenge,
18 mice were euthanized and lung, liver, kidney were collected for determination of
19 TNF- α and IL-6. Fig.4A₁-A₃: TNF- α in lung, liver and kidney; Fig.4 B₁-B₃: IL-6 in
20 lung, liver and kidney; Data presented are mean $\pm$ S.E.M. ^{##}P < 0.01 and ^{###}P < 0.001
21 vs. the control group; *P < 0.05 and **P < 0.01 vs. the LPS group.

22

1 **Fig.5.** Effects of esculin on tissue histopathological changes in LPS-induced
2 endotoxin shock mice (magnification $\times 100$). Tissues were stained with hematoxylin
3 and eosin for histopathological evaluation. Fig.5A₁-F₁, A₂-F₂, A₃-F₃: histopathological
4 changes of lung, liver and kidney in control group, LPS group, LPS + esculin (5, 10,
5 20 mg/kg) group, LPS + DEX (5 mg/kg) group, respectively; Fig.5G₁-G₃: injury
6 scores of lung, liver and kidney in different groups. Data presented are mean $\pm$ S.E.M.
7 ^{##}P < 0.01 and ^{###}P < 0.001 vs. the control group; *P < 0.05 and **P < 0.01 vs. the
8 LPS group.

9
10 **Fig.6.** Effects of esculin on immunohistochemical localization of NF- κ B p65 in
11 endotoxin shock mice (magnification $\times 100$). Fig.6A₁-F₁, A₂-F₂, A₃-F₃:: NF- κ B p65
12 protein expression in lung, liver and kidney in control group, LPS group, LPS +
13 esculin (5, 10, 20 mg/kg) group, LPS + DEX (5 mg/kg) group, respectively.
14 Fig.6G₁-G₃: mean density for NF- κ B p65 expression in different groups. Data
15 presented were mean $\pm$ S.E.M. ^{##}P < 0.01 and ^{###}P < 0.001 versus the control group;
16 *P < 0.05 and **P < 0.01 versus the LPS group.

17
18 **Fig.7.** Effects of esculin on protein expression of NF- κ B p65 in LPS-induced
19 endotoxin shock model. Animals were euthanized 6 h after LPS challenge, and the
20 tissue samples were harvested. NF- κ B p65 protein expression in lung, liver and
21 kidney were analyzed by western blot. Quantification of protein expression was
22 normalized to GAPDH using a densitometer. Similar results were observed in three

independent experiments and one of three representative experiments is shown. Data presented are mean $\pm$ S.E.M. $^{##}P < 0.01$ and $^{###}P < 0.001$ compared with the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the LPS group.

Fig.8. Effects of esculin on NO production in LPS-challenged macrophages. Macrophages were pretreated with different concentrations of esculin for 24 h before LPS stimulation (10 μ g/ml) for 12 h. Data presented are mean $\pm$ S.E.M. $^{##}P < 0.01$ when compared with the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared with the LPS group.

Fig.9. Effects of esculin on NF- κ B p65 protein expression in LPS-challenged macrophages. Cells were incubated with different concentrations of esculin (10^{-3} to 10^{-1} mg/ml) for 24 h before exposure to LPS (10 μ g/ml) for 12 h. Protein was extracted and then determined for NF- κ B p65 by Western blot analysis. One of three experiments is shown. The protein levels of NF- κ B p65 were normalized to GAPDH. $^{###}P < 0.001$ when compared with the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared with the LPS group.

Figure 1

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Figure 9

LPS(10μg/mL)	—	+	+	+	+
Esculin(mg/mL)	—	—	10 ⁻³	10 ⁻²	10 ⁻¹

Cortex Fraxini

Esculin