

# ***Total Flavonoids of Phellinus Igniarius Prevented Kidney Injury Induced by LPS***

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**Abstract.** Acute kidney injury (AKI) often occurs in septic patients, which can lead to permanent renal damage or ESRD (end-stage renal disease). Systemic and uncontrolled inflammation is a critical driver in the progression of sepsis-associated AKI. The current study aimed to investigate whether total flavonoids of *Phellinus igniarius* (TFPI) prevented lipopolysaccharide (LPS)-induced kidney injury. Male mice were treated with TFPI (8mg/kg) by gavage prior to LPS (15 mg/kg) administration. TFPI markedly decreased the level of plasma creatinine and gene expression of tubular injury markers (NGAL and Kim-1) in the kidney of mice with LPS, indicating an improved renal filtration ability and tubular injury. TFPI dramatically decreased the gene expression of MCP-1, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in the kidney of mice with LPS. TFPI also significantly downregulated protein abundance of TLR4, MyD88, p65 NF- $\kappa$ B, IL-1 $\beta$  and apoptosis proteins (Bax, Bcl-2 and cleaved caspase-3). In human proximal tubular cell line HK2 cells treated with LPS, TFPI markedly inhibited phosphorylation of p65 NF- $\kappa$ B and apoptosis. In conclusion, TFPI offered significant protection against LPS-induced kidney injury likely through mitigating inflammatory responses and apoptosis, supporting a treatment option of TFPI as a novel nephroprotective agent in managing sepsis-associated kidney injury.

**Keywords:** total flavonoids of *Phellinus igniarius*, LPS, kidney, inflammation

## **1. Introduction**

Sepsis-associated acute kidney injury (SA-AKI) is one of the most severe complications in patients with sepsis, which carries significantly increased mortality risks and poor prognosis [1]. In patients with sepsis, up to 60% may develop SA-AKI [2], current therapeutic strategies remain inadequate, identifying effective therapeutic targets are of great significance in the treatment of SA-AKI [1]. Although the molecular mechanism of SA-AKI has not been fully understood, uncontrolled inflammatory responses, mitochondrial dysfunction and multiple forms of programmed cell death have been recognized to contribute to the progression of SA-AKI [3].

Lipopolysaccharide (LPS) plays a potent initiator of the septic responses. LPS injection-induced AKI is the simplest experimental model of SA-AKI in rodents, which induces inflammation and mimicks the hyper-inflammatory state of sepsis [4]. LPS binds to Toll-like receptor 4 (TLR4) located on renal tubular epithelial cells, which recruits adaptor proteins (e.g. MyD88-dependent pathway), subsequently, induces release of multiple pro-inflammatory cytokines by activating NF- $\kappa$ B-dependent transcription [5-7], including tumor necrosis factor alpha (TNF- $\alpha$ ), monocyte chemoattractant protein-1 (MCP-1), and interleukins (IL-1 $\beta$ , IL-6). This cytokine surge causes interstitial infiltration, tubular epithelial apoptosis, mitochondrial swelling, and tubular epithelial degeneration [8, 9]. Therefore, targeting pro-inflammatory mediators may be a therapeutic option for attenuating kidney injury in SA-AKI. Apoptosis is one of hallmark features of LPS-induced SA-AKI. It occurs primarily in renal tubular epithelial cells where mitochondrial dysfunction and oxidative responses promote the activation of caspase-3, resulting in DNA fragmentation and cell death [10]. Apoptosis that occurs in renal tubular epithelial cells causes extensive loss of epithelial cells, leading to tubular atrophy and impaired regeneration during sepsis [10].

## 2. Literature review

The term flavonoid is a category of plant secondary metabolites which possess a polyphenolic structure and generate multiple flavonoid subclasses [11, 12]. Common flavonoids are easily found in vegetables and fruits. Plants with flavonoids had been used in Chinese traditional medicines to treat inflammation-related diseases for a long time. Over the past two decades, especially in the last five years, research on the renoprotective effects of flavonoids has grown rapidly. A number of in vivo animal experiments and in vitro cellular studies have confirmed that plant-derived flavonoid compounds possess kidney-protective biological activities [11, 13, 14]. Opuntiol [9], Hedyotis diffusa Willd [15], Luteolin [16], (-)-epicatechin [17], Quercetin [14], Vitisin A [18], Tectorigenin [19], Linarin [20] and Biochanin A [21] can effectively intervene in the development of sepsis-associated kidney injury. Mechanistically, flavonoids exert renoprotective effects through multiple synergistic pathways, mainly anti-oxidation and anti-inflammation.

*Phellinus igniarius* (PI) is shown to contain a variety of compounds, including polysaccharides, flavonoids, pyranones and furans, which have been demonstrated to possess anti-inflammatory, anti-oxidant and immuno-modulatory activities [22-24]. Total flavonoids extracted from *Phellinus igniarius* (TFPI) have been recently demonstrated to inhibit production of inflammatory cytokines (or chemokines) and suppress the expression of NF- $\kappa$ B [24, 25]. Unlike plant-derived flavonoids, the main active components of TFPI are dihydroflavones and dihydroflavonols (e.g. taxifolin), mostly present as free aglycones. They may differ significantly from common plant flavonoids (mostly flavonol glycosides) in chemical structure, lipophilicity, intestinal absorption, and preferential anti-inflammatory/antioxidant targets [22, 26, 27].

Wild PI is rare and low-yielding, which severely restricts research and industrial applications. Artificial cultivation is therefore essential to ensure stable supply and consistent quality of TFPI. At present, studies on the protection of TFPI against SA-AKI remain scarce, and the potential molecular mechanisms are largely unclear. Elucidating the renoprotective effects and mechanisms of TFPI against SA-AKI will not only reveal the unique structure–activity relationship and pharmacological features of fungal flavonoids, but also theoretically provide a molecular basis and experimental evidence for developing potent, novel and safe renoprotective functional ingredients in artificial cultivation.

Given this background, the current investigation aims to examine the protective effects and potential mechanism of TFPI in mice with SA-AKI induced by LPS and in vitro models. We

hypothesized that TFPI treatment would prevent kidney injury induced by LPS through suppressing pro-inflammatory cytokine expression, and tubular apoptosis.

### 3. Main body

#### 3.1. Materials and methods

##### 3.1.1. Animal study

C57BL/6 mice (Male, 8–10 weeks, 20–25g) were assigned randomly to three groups: control (CTL), LPS, and LPS + FPI (n = 6 in each group). Mice were injected LPS intraperitoneally (15 mg/kg) to induce AKI. Mice in the LPS + TFPI group were treated with TFPI by gavage (8 mg/kg) one hour prior to LPS treatment. Mice were sacrificed 24 hours after LPS administration.

##### 3.1.2. In vitro study

Human proximal tubular epithelial cells (HK2 cells) were cultured as described previously. LPS (10 µg/mL) was used to induce pro-inflammatory responses, with or without TFPI treatment (5 µg/mL).

##### 3.1.3. Measurement of protein and mRNA levels in cytokines and markers of apoptosis

Western blotting and q-PCR were used to measure the expression levels of protein or mRNA of cytokines and apoptosis markers in the kidney cortex or HK2 cells. HK2 cell apoptosis was assessed by using flowcytometry using Annexin VFITC/PI apoptosis detection kit.

##### 3.1.4. Statistical analysis

Data are presented as mean ± SEM. Differences among groups were analyzed by one-way ANOVA followed by an appropriate post hoc test. P < 0.05 was considered statistically significant.

### 3.2. Results

#### 3.2.1. TFPI improved renal filtration and attenuated tubular injury in mice with LPS

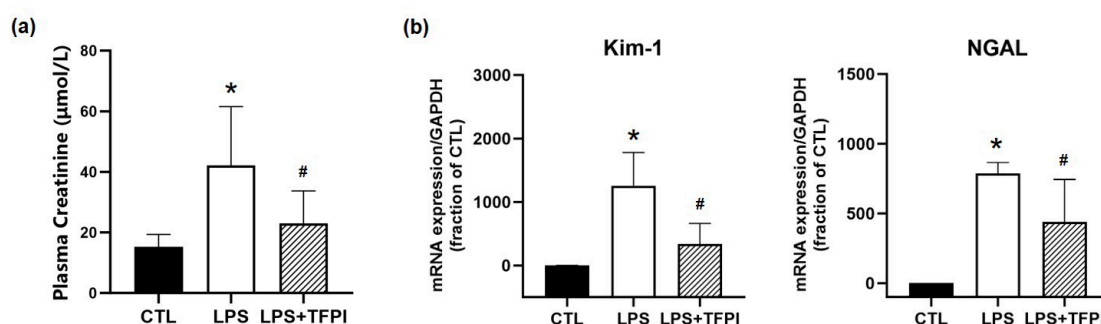


Figure 1. TFPI prevented LPS-induced filtration decline and tubular injury in mice. (a) Plasma creatinine levels in mice. (b) Analysis of mRNA levels by quantitative real-time PCR for Kim-1 and NGAL in the kidney of mice

Compared with controls, plasma creatinine level was dramatically increased in mice with LPS, while TFPI restored it to nearly control level (Figure 1A); LPS treatment caused marked upregulation of mRNA expression of Kim-1 and NGAL, two markers of tubular injuries, which was significantly inhibited by TFPI (Figure 1B). These data suggested that TFPI improved renal filtration ability and prevented tubular injury in mice with LPS.

### 3.2.2. TFPI inhibited inflammatory responses in the kidney of mice with LPS

Significant inflammatory responses were observed in cortex of the kidney of mice with LPS. mRNA levels of several pro-inflammatory factors were markedly upregulated in the kidney of mice treated with LPS, including TNF- $\alpha$ , MCP-1, IL-1 $\beta$  and IL-6, which were dramatically decreased by TFPI (Figure 2).

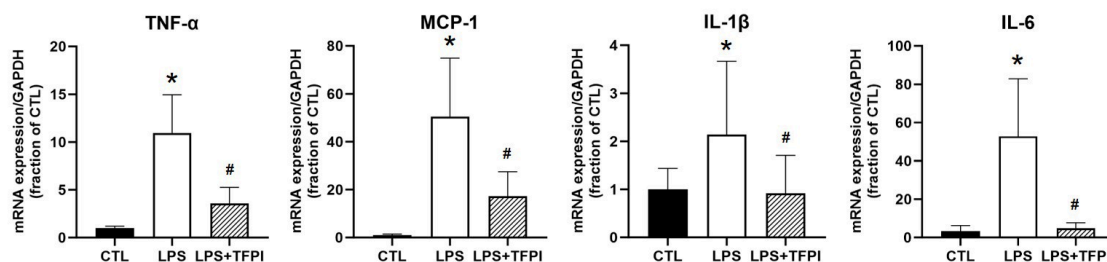


Figure 2. TFPI prevented LPS-induced inflammation in the kidney. Analysis of mRNA levels by quantitative real-time PCR for TNF- $\alpha$ , MCP-1, IL-1 $\beta$  and IL-6 in the kidney of mice

Consistent with alterations in mRNA expression levels, Western blotting demonstrated that the abundance of TLR4, MyD88, phosphorylated NF $\kappa$ B, and IL-1 $\beta$  protein was markedly upregulated, while TFPI at least partially inhibited these increases (Figure 3).

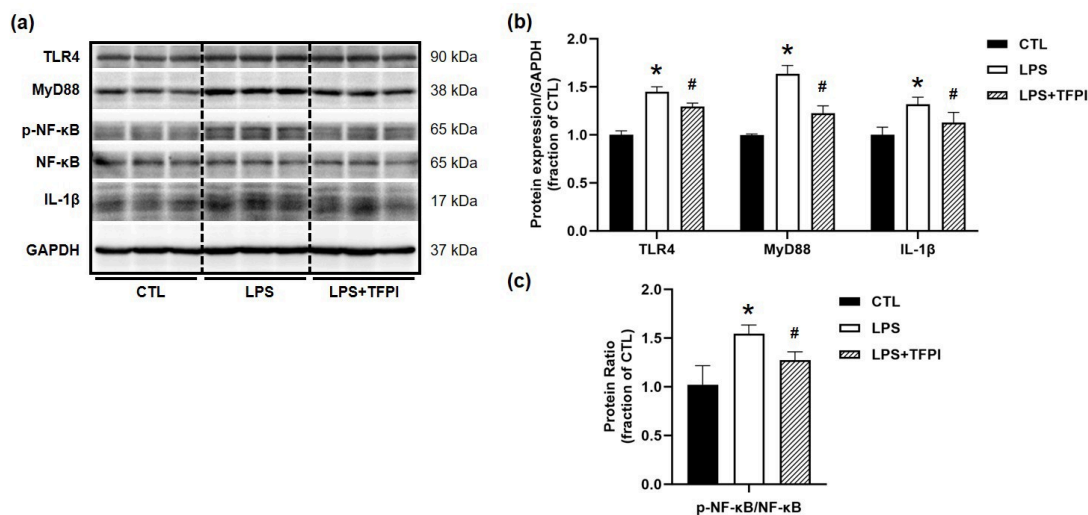


Figure 3. TFPI prevented LPS-induced inflammation in the kidney. (a) Semiquantitative immunoblots reacted with TLR4, MyD88, pNF- $\kappa$ B, NF- $\kappa$ B and IL-1 $\beta$ . (b) Corresponding densitometric analyses of protein levels of TLR4, MyD88 and IL-1 $\beta$  corrected by  $\beta$ -actin. (c) The ratio of protein abundance of pNF- $\kappa$ B p65/NF- $\kappa$ B

### 3.2.3. TFPI suppressed apoptosis in the kidney of mice with LPS

Compared with controls, the protein expression of proapoptotic markers Bax and caspase-3 was markedly upregulated in the kidney of mice with LPS, while anti-apoptotic protein Bcl-2 were much downregulated, all of these were significantly reversed by TFPI treatment (Figure 4). Taken together, these data support a role of TFPI as an agent that may suppress inflammation and apoptosis in LPS-induced AKI.

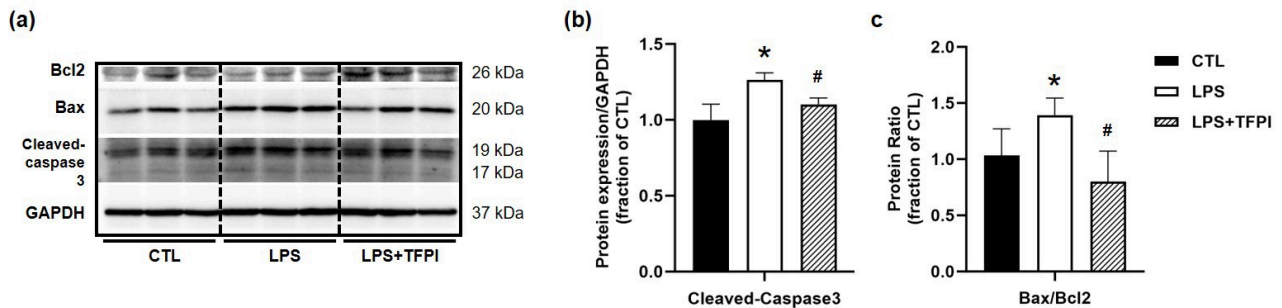


Figure 4. TFPI prevented LPS-induced apoptosis in the kidney. (a) Semiquantitative immunoblots reacted with Bax, Bcl-2 and cleaved caspase-1. (b) Corresponding densitometric analyses of protein levels of cleaved caspase-1 corrected by  $\beta$ -action. (c) The ratio of protein abundance of Bax/Bcl-2

### 3.2.4. TFPI suppressed inflammatory responses and apoptosis in LPS-treated HK2 cells

To better understand the protection of TFPI in SA-AKI, human kidney proximal tubule cell line HK2 cells were treated with LPS. LPS significantly induced the phosphorylation of NF $\kappa$ B, which was clearly inhibited by TFPI (Figure 5), potentially indicating an attenuated pro-inflammatory response. Flow cytometric analysis of apoptosis demonstrated that TFPI significantly suppressed LPS-induced apoptosis in HK2 cells (Figure 6).

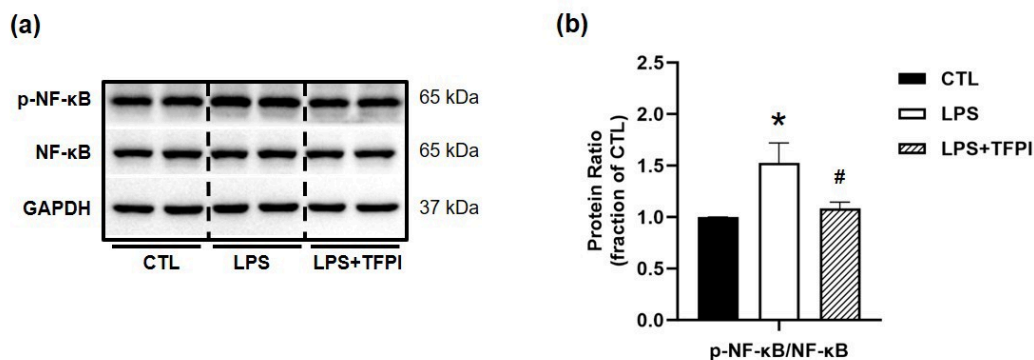


Figure 5. TFPI prevented LPS-induced pro-inflammatory response in HK2 cells. (a) Semiquantitative immunoblots reacted with pNF- $\kappa$ B p65 and NF- $\kappa$ B. (b) The ratio of protein abundance of pNF- $\kappa$ B p65/NF- $\kappa$ B

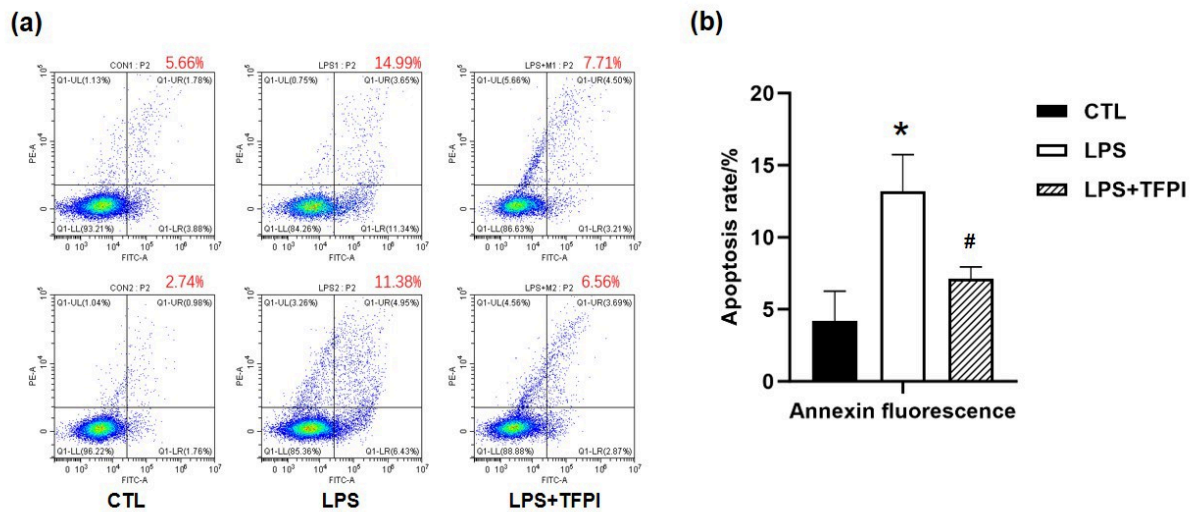


Figure 6. TFPI prevented LPS-induced apoptosis in HK2 cells. (a) Programmed cell death was detected by flow cytometry using Annexin VFITC/PI apoptosis detection kit in HK2 cells pretreated with TFPI, followed by LPS. (b) Corresponding analyses of apoptosis rate

#### 4. Discussion

In the current study, we demonstrated that TFPI, a naturally occurring flavonoid from *Phellinus igniarius*, effectively protected kidney from LPS-induced injury. TFPI effectively decreased plasma levels of creatinine and prevented increased mRNA expression of Kim-1 and NGAL, two markers of tubular injury, suggesting that TFPI significantly ameliorated renal filtration and tubular dysfunction caused by LPS challenge. The protection of TFPI is likely attributed to its properties of anti-inflammation and anti-apoptosis.

TLR4 signaling activation is critical in the pathogenesis of kidney injury induced by LPS. TLR4 activation promotes the overwhelming inflammatory responses [13] through NF- $\kappa$ B signaling [9] that is critical in the consequent expression of pro-inflammatory factors and the pro-apoptotic proteins [25]. Our study showed that TFPI markedly inhibited phosphorylation of NF- $\kappa$ B p65 in both mice and HK2 cells. The inhibition of NF- $\kappa$ B phosphorylation would cause decreased transcription of chemokines and cytokines. In the TLR4-MyD88-dependent pathway, TFPI effectively downregulated the protein expression of TLR4, MyD88, and IL-1 $\beta$  in the kidney of mice with LPS, which was also associated with decreased gene expression of several cytokines (e.g. TNF- $\alpha$ , MCP-1, IL-1 $\beta$  and IL-6), key drivers of tubular epithelial injury [28]. These data indicated that TFPI may disrupt inflammatory responses to pro-inflammatory stimuli or suppress the susceptibility of inflammatory cells to LPS. The inhibition of these cytokines may partially mitigate activation and adhesion of inflammatory cells to endothelial cells, hence reducing their infiltration and accumulation in inflamed regions [14, 17].

Not only inflammation, NF- $\kappa$ B activation can also modulate apoptosis. In the regulation of apoptosis, pro-apoptotic Bax can bind to anti-apoptotic Bcl-2 and antagonize the inhibitory effect of Bcl-2 on apoptosis. This is followed by the activation of caspase-3 that is responsible for the proteolytic degradation and apoptosis, the final execution [29]. Treatment with TFPI markedly decreased Bax and increased expression of Bcl, meanwhile, prevented the increase of cleaved-caspase-3 expression in the kidney of mice with LPS. These were further confirmed in vitro by flowcytometry measurement, in which TFPI significantly attenuated apoptosis of HK2 cells treated

with LPS. It is therefore suggested that TFPI protected kidney from LPS-induced injury, at least partially, through inhibiting tubular epithelial apoptosis.

In clinical transformation, traditional Chinese medicine and fungal extracts rich in flavonoids have provided new strategies for the adjuvant treatment of kidney diseases. TFPI is an excellent candidate, however, the in vivo metabolic characteristics, bioavailability and precise molecular targets of TFPI still need to be further clarified, which is also the key direction for in-depth research on the renoprotective effects of flavonoids represented by TFPI.

## 5. Conclusion

Our study provides preliminary evidence that TFPI could protect against the kidney injury induced by LPS, likely by inhibiting tubular inflammation and apoptosis. This study demonstrates that TFPI might be a potentially therapeutic option in the treatment of LPS-induced AKI.

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