Protection of Total Flavonoid Fraction from *Nervilia fordii* on Lipopolysaccharide-induced Acute Lung Injury in Rats

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Abstract: **Objective** To investigate the effects of total flavonoid fraction (TFF) from *Nervilia fordii* on lipopolysaccharide (LPS)-induced acute lung injury (ALI) in rats, and to explore their protective mechanism. **Methods** LPS-induced ALI model was established by LPS (5 mg/kg) injection via left cervical vein. Blood samples were collected from the cervical artery of all rats at 5 and 6 h after LPS challenge for arterial blood gas test and cytokines measurements, and pulmonary microvascular permeability (PMP), lung wet/dry weight ratio (W/D), and pathological features were observed. **Results** Phytochemical study showed that the TFF contained 67.3% of flavonoids expressed in rutin and three flavone glycosides. The TFF pretreatment (6.24 and 12.48 mg/kg) attenuated the partial arterial pressure of oxygen decline in blood significantly, and decreased the PMP and lung W/D in ALI rats. In addition, the TFF (6.24 and 12.48 mg/kg) also ameliorated the LPS-induced lung damages including alveolar edema, neutrophils infiltration, alveolar hemorrhage, and thickening of the alveolar wall. Furthermore, the treatment with the TFF (6.24 and 12.48 mg/kg) also down-regulated the levels of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and intercellular adhesion molecule-1 (ICAM-1), and up-regulated the level of anti-inflammatory cytokine IL-10 in serum of ALI rats simultaneously. **Conclusion** These results suggest that the TFF could protect LPS-induced ALI in rats, which may be mediated, at least in part, by adjusting the production of inflammatory cytokines including TNF-α, IL-6, ICAM-1, and IL-10.

Key words: acute lung injury; cytokine; *Nervilia fordii*; pulmonary microvascular permeability; total flavonoid fraction
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Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), the most severe stages of ALI, are complex syndromes of intense pulmonary inflammatory response with high morbidity and mortality (Tsushima *et al*, 2009). The main characteristics of ALI are the extensive neutrophil influx into lungs, the production of inflammatory mediators from inflammatory cells, and the damage to lung epithelial and endothelial surfaces leading to lung edema and impairment of respiratory function (Matuschak and Lechner, 2010; Piantadosi and Schwartz, 2004). Although great progresses have been made in pathophysiology of ALI, its treatment still remains to be a big challenge. Thus searching for new therapy targets and exploring new drugs to treat ALI would be valuable.

Nervilia fordii (Hance) Schltr., an orchidaceous plant endemic to the South of China, has long been used in Chinese folk medicine for the treatment of

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various respiratory diseases, such as bronchitis, stomatitis, acute pneumonia, and acute pharyngitis (Mei, 2008; Wu, 2003). Moreover, *N. fordii* was used as a folk medicine to treat severe acute respiration syndrome (SARS) in Guangdong Province during its epidemic outbreak (Feng, 2004; Wu, 2003). Our previous study confirmed that the crude extract of *N. fordii* could attenuate the development of pulmonary edema in ALI rats induced by lipopolysaccharide (LPS) (Xu, Chen, and Wang, 2010). However, the beneficial effects of *N. fordii* on ALI are mainly based on empirical experiences, and the fractions from this plant, which are responsible for the effects, have never been investigated.

In previous phytochemical studies on *N. fordii*, a series of flavonoids, triterpenes, and sterols have been reported. Among these compounds, flavonoids were suggested to be the main active ingredients with some pharmacological activities such as antiviral (Tian *et al*, 2009), anti-inflammatory (Zhou *et al*, 2009), and antitumor (Zhen *et al*, 2008) efficacies. The aim of this study was to investigate the protective effect of total flavonoid fraction (TFF) in *N. fordii* on ALI induced by LPS *in vivo* and to explore the possible mechanisms involved.

Materials and methods

Animals

Male Wistar rats (260—290 g) supplied by Center of Laboratory Animal of Guangzhou University of Chinese Medicine, were used in the experiments after acclimatization for 3 d. All rats were housed at an ambient temperature of (23 ± 1) ℃ and humidity of (55 ± 5)%. All experimental protocols were evaluated and approved by the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine.

Chemicals and plant materials

Lipopolysaccharide (LPS, *Escherichia coli*, O111: B4, No. 075k4036) and Evans blue dye (EBD) were obtained from Sigma Chemical Co. (USA). ELISA kits for tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1) were purchased from R & D Systems Inc. (USA).

Nervilia fordii (Hance) Schltr. was purchased from Chinese Herbal Medicines Market of Guangzhou (China), and identified by Prof. LAI Xiao-ping (Guangzhou University of Chinese Medicine). A voucher specimen has been deposited in Institute of Drug Research and Development, Guangzhou University of Chinese Medicine.

TFF preparation

The crushed herb of *N. fordii* (10 kg) was extracted twice with 50% ethanol for 1 h at 90 ℃. Two extracts were combined and concentrated under reduced pressure at 65 ℃, and then the distilled water was added to the extract. After being filtrated, the filtrate was chromatographed on AB-8 macroreticular resin (Nankai University Chemical Co., China) column using deionized water and 70% ethanol as eluant, respectively. The 70% ethanol fraction was concentrated under reduced pressure at 65 ℃, and then chromatographed on polyamide resin (60—80 meshes, Sinopec, China) column using the deionized water and 70% ethanol as eluant, respectively. The 70% ethanol fraction was concentrated under vacuum to give 31.2 g powder and was used as TFF.

Phytochemical identification of TFF

The TFF extract (200 mg) was dissolved in methanol-H_2O (2:1) and the solution was subjected to a high-performance counter-current chromatography instrument (TBE—300A, Tauto Biotech Co., Ltd., China). Three flavone glycosides were separated and the structures of the isolated compounds were further identified by comparison on their spectral data with those published references.

Determination of total flavonoids in TFF

The content of total flavonoids in TFF was determined using the method described in *Chinese Pharmacopoeia 2010* (Pharmacopoeia Committee of P. R. China, 2010). Briefly, TFF (25 mg) was dissolved in 10 mL of 70% ethanol and the solution (500 μL) was mixed with 1 mL of $NaNO_2$ (5%), 1 mL of 10% $AlCl_3$, 10 mL of NaOH (1 mol/L), and 12.5 mL of 70% ethanol. After incubation at room temperature for 20 min, the reaction mixture absorbance (*A*) was measured at 500 nm against a 70% ethanol blank on a spectrophotometer (Shimadzu UV—160A, Japan). Rutin (Tauto Biotech Co., Ltd., China) was chosen as a reference substance and the content of total flavor-

noids was expressed as milligram rutin equivalents (mg/g extract) through the calibration curve with rutin.

Establishment of ALI model and treatment regimen

The 48 rats were randomly divided into four groups with 12 rats in each group: control, LPS, and LPS + TFF (6.24 and 12.48 mg/kg) groups. TFF at doses of 6.24 and 12.48 mg/kg was ig administered once per day for consecutive 5 d, and the doses were equivalent to 2 and 4 g/kg of unprocessed *N. fordii*, which were on the basis of previous studies and our preliminary experiments. Rats from the control and LPS groups received the equal volume of distilled water instead of TFF.

LPS-induced ALI model was established as previous report with minor modifications (Fletcher *et al*, 1992). Briefly, 1 h after TFF treatment on day 5, the rats were anaesthetized by an ip injection of 10% chloral hydrate (3 mL/kg). The right carotid artery was cannulated with polyethylene-50 and connected to a pressure transducer (P231D, Statham, Oxnard, USA) for the measurement of arterial blood pressure and heart rate. The left cervical vein was cannulated for the administration of LPS. After all cardiovascular parameters were stabilized for 20 min, all rats except those in the normal control group received an iv injection of LPS (5 mg/kg). Blood samples were collected from the cervical artery of all rats at 5 and 6 h after LPS challenge for arterial blood gas test and cytokines measurements, respectively. Serum samples were obtained by centrifugation of whole blood and stored at −80 ℃ until assay.

Arterial blood gas analysis

For determining the levels of partial arterial pressure of oxygen (PaO_2) and partial arterial pressure of carbon dioxide ($PaCO_2$), blood samples (2 mL) were collected from the cervical artery of all rats at 5 h after LPS challenge and analyzed with a blood gas analyzer (Rapidlab, AVL-945, pH/Blood-gas Analyzer; Bayer, USA).

Measurement of pulmonary microvascular permeability and lung wet/dry weight ratio

Six rats were taken from each group and injected with 50 mg/kg EBD after blood sampling. After injection of EBD for 30 min, the rats were sacrificed immediately and the left lungs were removed and weighed. Formamide digests of the left lungs were prepared for 72 h at 45 ℃ and the homogenates were centrifuged at 12 000 × *g*. The *A* value was determined by measuring each sample at 620 nm and the ratio of *A*-lung weight was calculated representing pulmonary microvascular permeability (PMP) (Moitra, Sammani, and Garcia, 2007).

Following the previous experiment, the right lung was weighed and then dried to constant weight at 60 ℃ over 7 d in an oven. The dry weight was measured and the lung wet/dry weight ratio (W/D) was calculated.

Light microscopy observation

The rest rats in each group were sacrificed immediately after blood sampling and the left lungs were collected and fixed with an intratracheal instillation before processing for routine tissue slide, then stained with hematoxylin-eosin (HE) and observed under a light microscopy. All slides were coded and evaluated in a blinded fashion to prevent bias.

Scanning electron microscopy observation

A section of tissue 0.5 cm thick was excised from the right lung, cut into approximate 10 vertical slices, and trimmed into blocks. The blocks were fixed in 4% paraformaldehyde for 1 h, rinsed twice in 0.1 mol/L sodium phosphate, followed by 3% glutaraldehyde for 6 h. Then the specimens were fixed by 1% osmium tetroxide for 20 h, rinsed twice and followed by dehydration and embedment. The sections (50—70 nm) were cut and stained with saturated uranyl acetate and bismuth subnitrate. Microscopic examination was performed with a scanning electron microscope (SEM) (JEM 1200EX, JEOL, Japan).

Cytokines analysis

For determining the levels of inflammatory cytokines, such as TNF-α, IL-6, MCP-1, ICAM-1, and IL-10, blood samples (2 mL) were collected from the cervical artery of each rat and centrifuged at 3000 × *g* for 15 min to isolate the serum. The serum levels of cytokines were determined using commercially available ELISA kits according to the manufacturer's instructions.

Statistical analysis

The results were expressed as $\bar{x} \pm s$. Statistical significance of differences were evaluated by One-way

analysis of variance followed by Dunnett's *t* test (SPSS11.5). $P < 0.05$ was considered as statistical significance.

Results

Components in TFF

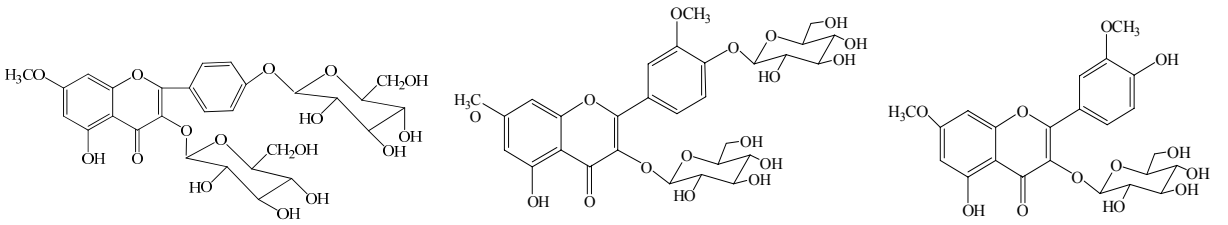
The content of total flavonoids in TFF was 67.3%, and three flavone glycosides (Fig. 1) were isolated and identified as complanatuside A, rhamnazin-3,4'-di-*O*-glucopyranoside, and rhamnazin-3-*O*-β-*D*-glucopyranoside, whose chemical structures were confirmed by comparison on their spectroscopic data including MS, ^{1}H-NMR, and ^{13}C-NMR with those in the literatures (Cui, Kinjo, and Nohara, 1993; Fukunaga *et al*, 1989; Thieme, 1968). The yield of these flavone glycosides from TFF was 6.5%, 25%, and 17.5%, respectively. Rhamnazin-3, 4'-di-*O*-glucopyranoside was reported from *N. fordii* for the first time.

Effect of TFF on LPS-induced hypoxemia

Compared with the control group, LPS injection could induce a significant decrease of PaO_2 but a significant increase of $PaCO_2$ TFF at 12.48 mg/kg could attenuate the LPS-induced PaO_2 decrease significantly, but fail to affect $PaCO_2$ levels (Table 1).

Effect of TFF on LPS-induced lung edema

To evaluate the effects of TFF on LPS-induced changes in pulmonary vascular permeability to water, PMP, and lung W/D were measured. Results showed that PMP and lung W/D increased significantly after LPS challenge compared with those in the control group. However, TFF at the doses of 6.24 and 12.48 mg/kg inhibited these LPS-induced increases in a dose-dependent manner significantly (Table 1).



complanatuside A rhamnazin-3,4'-di-*O*-glucopyranoside rhamnazin-3-*O*-β-*D*-glucopyranoside

Fig. 1 Chemical structures of three flavone glycosides isolated from TFF of *N. fordii*

Table 1 Effects of TFF pretreatment on respiratory index, PMP, and lung W/D in LPS-induced ALI rats ($\bar{x} \pm s$)

Groups	PaO_2 / mmHg	$PaCO_2$ / mmHg	PMP / ($A \cdot g^{-1}$)	lung W/D
control (distilled water)	95.6 ± 3.1	42.6 ± 2.9	1.30 ± 0.16	4.22 ± 0.10
LPS (distilled water)	67.2 ± 5.2$^{\#}$	54.4 ± 3.8$^{\#}$	2.09 ± 0.21$^{\#}$	5.02 ± 0.12$^{\#}$
LPS + TFF (6.24 $mg \cdot kg^{-1}$)	70.1 ± 4.4	51.3 ± 2.7	1.82 ± 0.19*	4.81 ± 0.11**
(12.48 $mg \cdot kg^{-1}$)	74.7 ± 3.1**	48.9 ± 3.4	1.65 ± 0.22**	4.70 ± 0.14**

$^{\#}P < 0.01$ *vs* control group; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* LPS group, same as below

Effect of TFF on LPS-induced histopathological change in lung tissues

Light microscopy results showed that the control group had normal pulmonary histology (Fig. 2A). However, the lung tissues in LPS group demonstrated obvious alveolar edema, neutrophils infiltration, and alveolar hemorrhage, as well as thickening of the alveolar wall (Fig. 2B). These morphologic changes were greatly improved after the treatment of TFF (Figs. 2C and 2D).

SEM results showed the normal histology of lungs in the control group (Fig. 3A). LPS injection not only resulted in the significant thickening of alveolar wall and massive fibrin formation, but also induced the loss of microvilli in bronchioles markedly (Fig. 3B), which could be partly reversed by TFF (Figs. 3C and 3D).

Effect of TFF on LPS-induced production of cytokines

The effects of TFF on TNF-α, IL-6, ICAM-1, MCP-1, and IL-10 production in serum of rats were analyzed by ELISA at 6 h after LPS challenge. Table 2

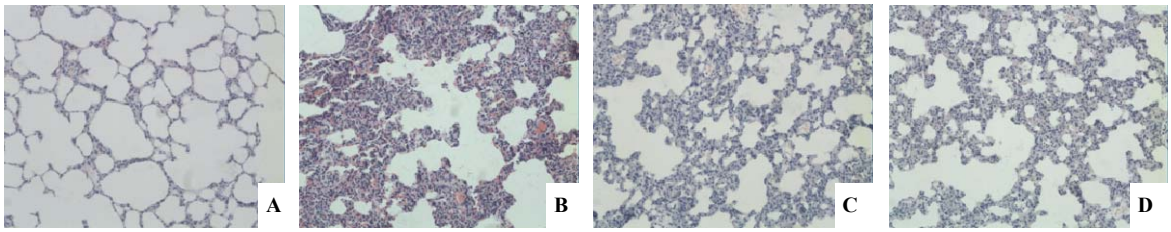


Fig. 2 Histological observation by light microscopy in control group (A), LPS group (B), LPS + TFF (6.24 mg·kg^{-1}) group (C), and LPS + TFF (12.48 mg·kg^{-1}) group (D) (n = 6)

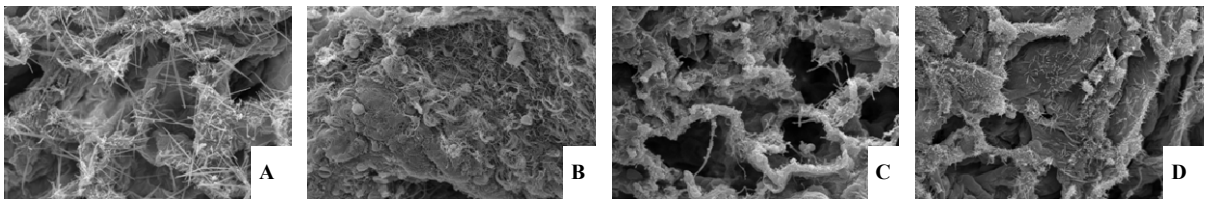


Fig. 3 Histological observation by SEM in control group (A), LPS group (B), LPS + TFF (6.24 mg·kg^{-1}) group (C), and LPS + TFF (12.48 mg·kg^{-1}) group (D) (n = 6)

Table 2 Effect of TFF pretreatment on serum levels of TNF-α, IL-6, IL-10, ICAM-1, and MCP-1 in LPS-induced ALI rats ($\bar{x} \pm s$)

Groups	TNF-α / (pg·mL^{-1})	IL-6 / (pg·mL^{-1})	MCP-1 / (pg·mL^{-1})	ICAM-1 / (pg·mL^{-1})	IL-10 / (ng·mL^{-1})
control (distilled water)	34.6 ± 8.4	144.3 ± 35.5	163.0 ± 29.7	21.8 ± 3.6	45.2 ± 14.3
LPS (distilled water)	782.6 ± 41.6$^{\#}$	1803.5 ± 247.0$^{\#}$	716.6 ± 46.3$^{\#}$	67.3 ± 6.7$^{\#}$	447.6 ± 46.5$^{\#}$
LPS + TFF (6.24 mg·kg^{-1})	285.6 ± 67.9*	1230.0 ± 155.5*	685.2 ± 33.1	53.3 ± 6.6*	585.8 ± 48.7*
(12.48 mg·kg^{-1})	316.8 ± 38.0*	1023.4 ± 128.0*	669.1 ± 42.5	46.2 ± 4.4*	632.0 ± 52.0*

showed that LPS injection induced the significant increase of TNF-α, IL-6, ICAM-1, MCP-1, and IL-10 levels in serum of rats. However, TFF decreased LPS-induced TNF-α, IL-6, and ICAM-1 production significantly, whereas enhanced IL-10 production.

Discussion and conclusion

Since Gram-negative bacteria-induced sepsis remains the most prevalent cause of ALI/ARDS in patients (Matthay *et al*, 2003), LPS was widely studied to elicit a systemic inflammatory response that re-sembled the clinical presentation of sepsis, including ALI and ARDS (Kabir *et al*, 2002). In the present study, the protective effects of TFF from *N. fordii* were investigated using the LPS-induced ALI rat model. Results showed that TFF could ameliorate hypoxemia, decrease lung edema, attenuated lung damage, and reduce the extent of inflammation in ALI rat model. To the best of our knowledge, this is the first report about the protective effect of TFF on LPS-induced lung injury. Moreover, we found that the protection of TFF on LPS-induced ALI seemed to be associated with their influence on varied inflammatory cytokines release, including pro-inflammatory cytokine TNF-α, IL-6, and ICAM-1, and anti-inflammatory cytokine IL-10.

In LPS-induced ALI rats, changes in the lungs including hypoxemia become evident within 2—4 h after iv administration of LPS (Matute-Bello, Frevert, and Martin, 2008). As expected, LPS injection induced a significant decrease of PaO_2 while a significant increase of $PaCO_2$, indicating the existence of obvious hypoxemia. Pretreatment of TFF inhibited the LPS-induced PaO_2 decline significantly, which suggested that it might ameliorate lung dysfunction and improve gas exchange.

Edema is a typical symptom of inflammation not only in systemic inflammation, but also in local inflammation. The lung W/D showed that pretreatment of TFF decreased the lung W/D significantly, indicating that TFF could inhibit the leakage of serous fluid into lung tissue and attenuate the development of lung edema. As another index of ALI induced by LPS, the increased PMP to fluid and protein causes lung edema and ALI (Staub, 1978). As expected, rats exposed to LPS presented with significantly increased PMP.

LPS-induced increases in PMP were inhibited by TFF.

LPS is known to alter the production of several cytokines, such as TNF-α, IL-6, ICAM-1, and IL-10, which have been demonstrated to provoke life-threatening conditions, including ALI/ARDS and multiorgan failure (Bhatia and Moochhala, 2004; Ware and Matthay, 2000). TNF-α and IL-6, two early pro-inflammatory cytokines, play critical roles in the pathogenesis of ALI and contribute to the severity of lung injury (Giebelen *et al*, 2007). ICAM-1 is a key cell adhesion molecule and the severity of the lung injury correlates well with the expression of ICAM-1 protein (Frossard *et al*, 1999). In addition, anti-inflammatory cytokine IL-10 is vitally important for modulating the inflammatory response to microbial products (Latifi, O'Riordan, and Levine, 2002) and IL-10 treatment *in vivo* could protect the lungs against LPS-induced injury (Wu *et al*, 2009). In this study, the concentration of TNF-α, IL-6, ICAM-1, and IL-10 in blood increased significantly after LPS challenge and the pretreatment of TFF attenuated the LPS-induced increases of TNF-α, IL-6, and ICAM-1 markedly, whereas enhanced the production of IL-10 simultaneously. Therefore, TFF may protect lung tissues against LPS-induced ALI by adjusting the production of these pro-inflammatory and anti-inflammatory cytokines.

Flavonoids have shown multiple bioactivities including anti-inflammation (Kim *et al*, 2004). In this study, the total flavonoids account for 67.3% in TFF, complanatuside A, rhamnazin-3,4'-di-*O*-glucopyranoside, and rhamnazin-3-*O*-β-*D*-glucopyranoside were isolated and identified. Although the anti-inflammatory effects of these components have not been established, their aglycone rhamnazins showed the potent anti-inflammatory activities which were even higher than these of the positive control mefenamic acid (Martini, Katerere, and Eloff, 2004). Besides, previous studies have shown that several other aglycones from *N. fordii*, e.g. rhamnocitrin and rhamnetin, showed significant anti-inflammatory activity (Bucar *et al*, 1998; Fang *et al*, 2008). Thus, these isolated flavonoids could partially explain the observed beneficial activity of TFF. Further studies are required to investigate the potential effects of these compounds and the possible mechanism needs to be determined.

In conclusion, the present study demonstrats that TFF from *N. fordii* shows protective effect against LPS-induced ALI in rats, which suggests that the flavornoids may be the major effective fraction of *N. fordii*. Furthermore, this study may provide new evidence for the traditional application of *N. fordii* in the treatment of respiratory diseases.

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