



## Original article

# Antipyretic Effects of Liposoluble Fractions of *Viola yedoensis*

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### ABSTRACT

**Objective** To clarify the antipyretic effect of the Chinese materia medica, *Violae Herba* (*Viola yedoensis*), and its active fractions by examining the effects of *V. yedoensis* extracts with differing polarities on body temperature, total white blood cell (WBC) count, WBC differential count, and total serum complement of rabbits with lipopolysaccharide (LPS)-induced fever. **Methods** The rabbits were treated with water and ethanolic extracts of *V. yedoensis*, as well as petroleum ether, ethyl acetate, and *n*-butanol fractions of the ethanolic extract at low-, mid- and high- doses. The LPS was injected via the ear vein of rabbits in model and treatment groups 30 min post-gavage. Their body temperature was measured at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 h after the LPS challenge to calculate the temperature changes and thermal response index. After the last temperature measurement, blood samples were collected to determine the blood cell counts and total serum complement (CH<sub>50</sub>) level. **Results** Compared with the model group, body temperature was significantly lower in the low-dose ethanolic extract group, low- and mid-dose petroleum ether fraction groups, and all three ethyl acetate fraction groups. Serum CH<sub>50</sub> levels were lower in all treatment groups, except the ethanolic extract groups, than that in the model group, with no significant difference. *V. yedoensis* had no significant effect on the blood cells of febrile rabbits challenged with LPS for 6 h. **Conclusion** The antipyretic effects of *V. yedoensis* are strong, and its active fractions are the petroleum ether and ethyl acetate fractions of ethanolic extract.

#### Key words

anti-complement; antipyretic effect; heat-clearing; *Viola yedoensis*

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## 1. Introduction

*Violae Herba* is a Chinese materia medica for clearing away heat and toxic materials. It is the dried whole plant of the perennial herb, *Viola yedoensis* Makino (Family Violaceae) (Pharmacopoeia Committee of P. R. China, 2010). It has the

effects of clearing away heat, detoxicating, cooling blood, and subduing swellings. In clinical practice, it is mainly used for furunculosis, sores, swellings, ulcers, erysipelas, acute mastitis, and acute appendicitis, as well as other suppurative and infectious diseases. Modern pharmacological studies have found that *V. yedoensis* has anti-inflammatory, antibacterial,

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anti-HIV, immunomodulating, and antihyperuricemic effects. For example, Chen et al (2008) and Kang (2012) demonstrated that the decoction and ethyl acetate (EA) fraction of the ethanolic extract of *V. yedoensis* had a strong inhibitory effect on *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, and other bacteria. In an antiviral study, eight cyclotides separated from *V. yedoensis* were shown to have anti-HIV activity *in vitro* (Wang et al, 2008). However, it is worth noting that the antibacterial and antiviral activity of *V. yedoensis* is difficult to reproduce. Our previous attempts failed to demonstrate a significant antibacterial or anti-HIV activity with the water and ethanolic extracts and fractions of differing ethanolic extract polarities (data not shown). Nonetheless, *V. yedoensis* indeed had a high anti-inflammatory activity; For example, its decoction and ethanolic extract significantly inhibited xylene-induced mouse ear swelling (Chen et al, 2008). Moreover, we recently proved that a petroleum ether (PE) fraction of the ethanolic extract of *V. yedoensis* had a significant therapeutic effect on lipopolysaccharide (LPS)-induced acute lung injury in mice; The results suggested that it might reduce acute lung injury by reducing pulmonary capillary permeability and pulmonary edema and inhibiting a local inflammatory response through its anti-inflammatory mechanism (Li et al, 2012). The anti-inflammatory activity may be related to its immunomodulating action, for example, Li and Hu (2012) reported that the anti-inflammatory effects of the water and ethanolic extracts of *V. yedoensis* might be associated with the decreased expression of TNF- $\alpha$ , IL-1 $\beta$ , and PGE<sub>2</sub>. It is not yet known whether *Violae Herba* has a definite antipyretic effect as a heat-clearing drug and whether this effect is one of the mechanisms for its treatment of acute lung injury. Therefore, this study aimed to examine the antipyretic effect of *V. yedoensis* and its active substances, in order to provide a systematic exposition of the heat-clearing effect of *Violae Herba* based on the changes in white blood cell (WBC) count and complement level.

## 2. Materials and methods

### 2.1 Chemicals and reagents

Water extract (0.61%) and ethanolic extract (10.47%) of *Viola yedoensis* Makino and PE (3.07%), EA (1.30%), and *n*-butanol (BU, 0.60%) fractions of the ethanolic extract were self-made (Li et al, 2012). Aspirin (Nanjing Baijinyu Pharmaceutical Co. Ltd., China), *Escherichia coli* O111B4 LPS (ET; Sigma-Aldrich, USA), sodium pentobarbital (Sigma), dimethylsulfoxide (DMSO), sodium carboxymethyl cellulose (CMC-Na), and ELISA kit for the total complement (CH<sub>50</sub>) of the rabbit serum (Meilian Bio-Tech Co. Ltd., Shanghai, China) were used.

### 2.2 Experimental animals

One-hundred and forty New Zealand white rabbits, male or female, weighing (2.5  $\pm$  0.2) kg (Shengwang Laboratory

Animal Co. Ltd., China; license No. SCXK (Shanghai) 2007-0007) were used. The animals were housed at room temperature of 16–26 °C with humidity of 40%–70%.

### 2.3 Equipments

Ordinary household mercury thermometer, ADVIA 120 Hematology Analyzer with a five-part differential capability (Bayer AG, Germany), Synergy HT Multidetector Microplate Reader (BioTek, VT), and catheters for human were used.

### 2.4 Drug solution preparation

We prepared the stock solution (100 mg/mL) to be tested as follows: 2 g water and the ethanolic extracts of *V. yedoensis* and fractions of each ethanolic extract were added to 200  $\mu$ L DMSO and dissolved in 20 mL water with ultrasound. The resulting solutions were transferred into tubes (5 mL each) and stored at –70 °C. They were diluted with distilled water into low-dose (2 mg/mL), mid-dose (6 mg/mL), and high-dose (10 mg/mL) solutions just before use. The control group was given a water solution containing 0.1% DMSO (same as high-dose solution). Preparation of aspirin suspension (20 mg/mL) was performed as follows: one aspirin tablet (0.4 g) was ground, and 0.5% CMC-Na was added to achieve 20-mL volume. After mixing, 0.1% DMSO was added to the mixture. Preparation of LPS solution (0.25  $\mu$ g/mL) was then performed as follows: 10 mg LPS was diluted with 50 mL nonpyrogenic saline, added to 0.2 mg/mL stock solution, and stored at 4 °C for later use. The stock solution was ultrasonically treated for 20 min, and 40 mL normal saline was added to 50  $\mu$ L stock solution and mixed just before use.

### 2.5 Selection and screening of eligible rabbits

Healthy and eligible rabbits were selected, and their temperature was measured daily for consecutive 3 d. Rabbits with the temperature of 39.6–40.0 °C and a fluctuation of less than 0.3 °C were finally selected for the experiment.

One-hundred and eight eligible rabbits were randomly divided into 18 groups ( $n = 6$ ), such as normal group, model group, positive control (aspirin) group, as well as low-, mid-, and high-dose PE fraction of *V. yedoensis* ethanolic extract groups; low-, mid-, and high-dose EA fractions groups; low-, mid-, and high-dose BU fraction groups; and low-, mid-, and high-dose *V. yedoensis* water extract groups. The low-, mid-, and high doses were set at 10, 30, and 50 mg/kg, respectively.

### 2.6 Body temperature measurement of rabbits with an LPS-induced fever

The body temperature of each rabbit was measured twice before the experiment at a 20-min interval, and the average was used as the basal body temperature before administration. The rabbits in each treatment group were ig administered with an equal volume (5 mL/kg) of corresponding medication, and

the rabbits in the normal and model groups were given the same volume of distilled water. After 30 min, 1 mL/kg (equal to 0.25 µg/kg of a mass unit) LPS was iv injected into ear vein of each rabbit, except for the normal group. The time point when the injection was completed was considered to be 0 h, and the body temperature of rabbits was measured at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 h. The changes in the body temperature of rabbits at each time point were calculated by using the basal body temperature as a base.

### 2.7 Blood cell measurement of rabbits with LPS-induced fever

Rabbits in each group were anesthetized by ear vein injection of 30 mg/kg sodium pentobarbital (3%) after the last temperature measurement. Blood samples were collected from heart and anticoagulated with EDTA-2K. WBC and platelet (PLT) counts were determined in hematology analyzer.

### 2.8 Total complement measurement of serum of rabbits with LPS-induced fever

Serum samples were collected from each rabbit. The level of total complement (CH<sub>50</sub>) in the serum was then measured using the ELISA kit for CH<sub>50</sub> in the serum of rabbit, along with the Synergy HT Multidetector Microplate Reader.

### 2.9 Statistical analysis

All data were analyzed using SPSS 13.0 software. For a mean comparison among the groups, analysis of variance (ANOVA) was used if the variance was homogeneous, and a non-parametric test was applied if it was nonhomogeneous.

## 3. Results

### 3.1 Chemical constituents from different fractions of ethanolic extracts

The chemical constituents in PE fraction were previously studied by GC-MS (Li et al, 2012a) and the main components were hexadecanoic acid ethyl ester (24.87%), (Z,Z,Z)-9,12,15-octadecatrienolic acid ethyl ester (17.86%), 9,12-octadecadienolic acid ethyl ester (13.29%), (Z,Z,Z)-9,12,15-octadecatrienolic acid (13.25%), *n*-hexadecanoic acid (12.02%), and phytol (5.86%). The chemical constituents in the EA fraction were studied by traditional column chromatography. A number of compounds such as triterpenoids, alkaloids, coumarins, and phenolic acids have been isolated and identified (unpublished data). Recently, the chemical profile of the BU fraction was investigated by HPLC-ion trap mass spectrometry, and 32 flavone C-glycosides and three flavonol *O*-glycosides were characterized (Cao et al, 2014).

### 3.2 Effects of each *V. yedoensis* extract on temperature changes of rabbits with LPS-induced fever

The increase in the body temperature was higher in the model group than that in the normal group at each time point from 0.5 h to 5.5 h after the ear vein injection of 0.25 µg/kg LPS, and the differences were statistically significant ( $P < 0.05$  or  $0.01$ ), indicating that the model was successfully established in this experiment. In addition, there were two peaks in the temperature curve. The first peak occurred at 0.5 h and the second occurred at 3.0 h, with an observed greatest increase of 1.12 °C. Their temperature tended to be normal at 6.0 h. The increase in the body temperature was smaller in the low- and mid-dose *V. yedoensis* water extract groups within 3.0 h of the LPS challenge and in the high-dose group within 6.0 h, compared with that in the model group. However, the differences were not statistically significant, which indicated that the water extract of *V. yedoensis* had no significant effect on their body temperature (Table 1).

The increase in the body temperature was significantly reduced in low- and mid- dose *V. yedoensis* ethanolic extract groups from 1.0 h to 2.0 h after LPS challenge ( $P < 0.05$  or  $0.01$ ). In particular, the thermal response index (TRI) in the low-dose group was significantly different from that in the model group ( $P < 0.05$ ). Although the increase in body temperature was reduced in the high-dose group, the difference was not statistically significant (Table 1).

The above-mentioned findings suggested that the antipyretic effect of *V. yedoensis* was produced by its ethanolic extract. In order to better understand the antipyretic effect of the active constituents, the antipyretic effect of the PE, EA, and BU fractions of the ethanolic extract were further investigated.

The increase in the body temperature was smaller in each dose group than that in the model group at 0.5 h after ig administration of *V. yedoensis* PE fraction ( $P < 0.05$ ). The increase in the body temperature was significantly smaller in the low-dose group than that in the model group from 1.0 h to 3.0 h after administration ( $P < 0.01$  or  $0.05$ ), and it was significantly smaller in the mid-dose group than that in the model group from 1.0 h to 3.5 h after administration ( $P < 0.01$ ). Compared with the model group, the TRI was significantly decreased in the low- and mid- dose groups ( $P < 0.01$  or  $0.05$ ). These results suggested that the low- and mid-dose *V. yedoensis* PE fraction could effectively inhibit the increase of the body temperature in rabbits with LPS-induced fever and demonstrate the significant antipyretic effect (Table 2).

As compared with the model group, the increase in the body temperature was significantly smaller in the mid- and high-dose *V. yedoensis* EA fraction groups at 0.5 h after the LPS challenge, in each dose group between from 1.0 h to 3.0 h, and in the low- and mid- dose groups between 3.5–4.0 h (all  $P < 0.01$  or  $0.05$ ). The TRI was also significantly lower in each dose group than in the model group. The results demonstrated that the three EA fraction dose levels all had a significant antipyretic effect (Table 2).

**Table 1** Effects of water and ethanolic extracts from *V. yedoensis* on temperature changes and TRI of rabbits with fever ( $\bar{x} \pm s, n = 6$ )

Groups	Doses / (mg·kg <sup>-1</sup> )	Temperature change / °C						
		0.5 h	1.0 h	1.5 h	2.0 h	2.5 h	3.0 h	3.5 h
normal	–	-0.08 ± 0.44	-0.25 ± 0.31	-0.45 ± 0.38	-0.28 ± 0.66	-0.63 ± 0.23	-0.58 ± 0.23	-0.62 ± 0.57
model	–	0.75 ± 0.19 <sup>▲▲</sup>	0.40 ± 0.13 <sup>▲▲</sup>	0.93 ± 0.23 <sup>▲▲</sup>	0.92 ± 0.23 <sup>▲▲</sup>	1.02 ± 0.25 <sup>▲▲</sup>	1.12 ± 0.26 <sup>▲▲</sup>	0.98 ± 0.32 <sup>▲▲</sup>
positive control	–	0.13 ± 0.39 <sup>**</sup>	0.45 ± 0.63	0.38 ± 0.53 <sup>*</sup>	0.42 ± 0.69	0.53 ± 0.76	0.67 ± 0.87	0.77 ± 0.78
<i>V. yedoensis</i>	10	0.40 ± 0.28	0.62 ± 0.39	0.58 ± 0.44	0.63 ± 0.62	0.83 ± 0.56	0.97 ± 0.48	1.03 ± 0.28
water extract	30	0.53 ± 0.32	0.78 ± 0.48	0.70 ± 0.41	0.82 ± 0.62	0.85 ± 0.60	1.03 ± 0.71	1.08 ± 0.77
	50	0.67 ± 0.31	0.77 ± 0.36	0.68 ± 0.48	0.70 ± 0.70	0.83 ± 0.84	0.92 ± 0.93	0.77 ± 0.85
<i>V. yedoensis</i>	10	0.43 ± 0.39	0.38 ± 0.31 <sup>**</sup>	0.30 ± 0.36 <sup>**</sup>	0.27 ± 0.60 <sup>*</sup>	0.40 ± 0.65	0.42 ± 0.60 <sup>*</sup>	0.42 ± 0.68
ethanolic	30	0.30 ± 0.58 <sup>*</sup>	0.28 ± 0.53 <sup>*</sup>	0.03 ± 0.62 <sup>*</sup>	-0.03 ± 0.85 <sup>*</sup>	0.13 ± 0.98	0.17 ± 1.02	0.08 ± 1.02
extract	50	0.18 ± 0.65 <sup>*</sup>	0.37 ± 0.76	0.32 ± 0.66	0.33 ± 0.75	0.55 ± 0.91	0.33 ± 0.86	0.40 ± 1.00

Groups	Doses / (mg·kg <sup>-1</sup> )	Temperature change / °C					TRI / (°C·h)
		4.0 h	4.5 h	5.0 h	5.5 h	6.0 h	
normal	–	-0.60 ± 0.33	-0.53 ± 0.39	-0.41 ± 0.50	-0.53 ± 0.48	-0.37 ± 0.58	-5.13 ± 3.16
model	–	0.92 ± 0.44 <sup>▲▲</sup>	0.68 ± 0.45 <sup>▲▲</sup>	0.30 ± 0.41 <sup>▲</sup>	0.10 ± 0.37 <sup>▲</sup>	-0.22 ± 0.37	8.27 ± 3.07 <sup>▲▲</sup>
positive control	–	0.62 ± 0.68	0.37 ± 0.68	-0.02 ± 0.66	-0.35 ± 0.64	-0.50 ± 0.57	3.65 ± 7.09
<i>V. yedoensis</i>	10	0.97 ± 0.36	0.73 ± 0.36	0.43 ± 0.49	0.32 ± 0.48	0.17 ± 0.52	7.40 ± 3.38
water extract	30	1.00 ± 0.52	0.90 ± 0.43	0.43 ± 0.45	0.22 ± 0.34	0.12 ± 0.39	8.14 ± 4.81
	50	0.57 ± 0.70	0.37 ± 0.59	0.07 ± 0.47	-0.18 ± 0.35	-0.37 ± 0.36	5.63 ± 5.97
<i>V. yedoensis</i>	10	0.28 ± 0.73	0.12 ± 0.47	-0.12 ± 0.43	-0.32 ± 0.45	-0.38 ± 0.58	2.18 ± 4.97 <sup>*</sup>
ethanolic	30	0.05 ± 1.00	0.02 ± 0.80	-0.12 ± 0.78	-0.27 ± 0.73	-0.40 ± 0.66	0.30 ± 8.11
extract	50	0.37 ± 0.84	0.18 ± 0.71	0.00 ± 0.56	-0.17 ± 0.43	-0.42 ± 0.40	2.57 ± 7.42

▲*P* < 0.05 ▲▲*P* < 0.01 vs normal group; \**P* < 0.05 \*\**P* < 0.01 vs model group; same as below

**Table 2** Effects of ethanolic extract fractions from *V. yedoensis* on temperature changes and TRI of rabbits with fever ( $\bar{x} \pm s, n = 6$ )

Groups	Doses / (mg·kg <sup>-1</sup> )	Temperature change / °C						
		0.5 h	1.0 h	1.5 h	2.0 h	2.5 h	3.0 h	3.5 h
normal	–	-0.08 ± 0.44	-0.25 ± 0.31	-0.45 ± 0.38	-0.28 ± 0.66	-0.63 ± 0.23	-0.58 ± 0.23	-0.62 ± 0.57
model	–	0.75 ± 0.19 <sup>▲▲</sup>	0.40 ± 0.13 <sup>▲▲</sup>	0.93 ± 0.23 <sup>▲▲</sup>	0.92 ± 0.23 <sup>▲▲</sup>	1.02 ± 0.25 <sup>▲▲</sup>	1.12 ± 0.26 <sup>▲▲</sup>	0.98 ± 0.32 <sup>▲▲</sup>
positive control	–	0.13 ± 0.39 <sup>**</sup>	0.45 ± 0.63	0.38 ± 0.53 <sup>*</sup>	0.42 ± 0.69	0.53 ± 0.76	0.67 ± 0.87	0.77 ± 0.78
PE Fraction	10	0.24 ± 0.23 <sup>*</sup>	1.03 ± 0.31 <sup>**</sup>	0.38 ± 0.19 <sup>**</sup>	0.10 ± 0.41 <sup>**</sup>	0.25 ± 0.31 <sup>**</sup>	0.38 ± 0.64 <sup>*</sup>	0.35 ± 0.72
	30	0.18 ± 0.32 <sup>*</sup>	0.10 ± 0.41 <sup>**</sup>	0.12 ± 0.29 <sup>**</sup>	0.05 ± 0.34 <sup>**</sup>	0.10 ± 0.43 <sup>**</sup>	0.18 ± 0.44 <sup>**</sup>	0.30 ± 0.30 <sup>**</sup>
	50	0.27 ± 0.33 <sup>*</sup>	0.65 ± 0.49	0.63 ± 0.52	0.68 ± 0.69	0.85 ± 0.79	0.98 ± 0.79	1.02 ± 0.56
EA Fraction	10	0.38 ± 0.23	0.33 ± 0.37 <sup>**</sup>	0.27 ± 0.45 <sup>**</sup>	0.07 ± 0.43 <sup>**</sup>	0.08 ± 0.59 <sup>**</sup>	0.17 ± 0.61 <sup>**</sup>	0.17 ± 0.42 <sup>**</sup>
	30	0.02 ± 0.36 <sup>**</sup>	0.33 ± 0.66 <sup>*</sup>	-0.02 ± 0.34 <sup>**</sup>	-0.03 ± 0.53 <sup>**</sup>	0.23 ± 0.42 <sup>**</sup>	0.47 ± 0.38 <sup>**</sup>	0.43 ± 0.42 <sup>*</sup>
	50	0.22 ± 0.44 <sup>*</sup>	0.27 ± 0.38 <sup>**</sup>	0.18 ± 0.50 <sup>**</sup>	0.15 ± 0.65 <sup>*</sup>	0.37 ± 0.58 <sup>*</sup>	0.33 ± 0.69 <sup>*</sup>	0.60 ± 0.43
BU Fraction	10	0.30 ± 0.17 <sup>*</sup>	0.48 ± 0.23 <sup>**</sup>	0.45 ± 0.49	0.50 ± 0.38 <sup>*</sup>	0.60 ± 0.52	0.57 ± 0.62	0.42 ± 0.62
	30	0.40 ± 0.41	0.57 ± 0.52	0.67 ± 0.82	0.45 ± 0.95	0.63 ± 1.09	0.78 ± 0.97	0.87 ± 0.88
	50	0.25 ± 0.54 <sup>*</sup>	0.22 ± 0.81	-0.05 ± 1.04	0.05 ± 1.50	0.15 ± 1.11	0.08 ± 1.42	0.03 ± 1.39

Groups	Doses / (mg·kg <sup>-1</sup> )	Temperature change / °C					TRI / (°C·h)
		4.0 h	4.5 h	5.0 h	5.5 h	6.0 h	
normal	–	-0.60 ± 0.33	-0.53 ± 0.39	-0.41 ± 0.50	-0.53 ± 0.48	-0.37 ± 0.58	-5.13 ± 3.16
model	–	0.92 ± 0.44 <sup>▲▲</sup>	0.68 ± 0.45 <sup>▲▲</sup>	0.30 ± 0.41 <sup>▲</sup>	0.10 ± 0.37 <sup>▲</sup>	-0.22 ± 0.37	8.27 ± 3.07 <sup>▲▲</sup>
positive control	–	0.62 ± 0.68	0.37 ± 0.68	-0.02 ± 0.66	-0.35 ± 0.64	-0.50 ± 0.57	3.65 ± 7.09
PE fraction	10	0.27 ± 0.80	-0.02 ± 0.90	-0.23 ± 0.93	-0.45 ± 0.87	-0.58 ± 0.81	1.26 ± 5.29 <sup>*</sup>
	30	0.35 ± 0.51	0.17 ± 0.54	-0.03 ± 0.55	-0.17 ± 0.50	-0.30 ± 0.39	1.11 ± 3.97 <sup>**</sup>
	50	0.72 ± 0.52	0.35 ± 0.51	0.08 ± 0.32	-0.22 ± 0.29	-0.30 ± 0.29	5.73 ± 5.56
EA fraction	10	0.13 ± 0.41 <sup>*</sup>	0.00 ± 0.33	-0.10 ± 0.38	-0.25 ± 0.31	-0.25 ± 0.34	0.93 ± 2.78 <sup>**</sup>
	30	0.20 ± 0.34 <sup>*</sup>	0.12 ± 0.31	-0.22 ± 0.34	-0.42 ± 0.36	-0.55 ± 0.29	0.83 ± 3.19 <sup>**</sup>
	50	0.57 ± 0.44	0.18 ± 0.61	0.05 ± 0.67	-0.12 ± 0.56	-0.20 ± 0.54	2.59 ± 4.62 <sup>*</sup>
BU fraction	10	0.40 ± 0.68	0.27 ± 0.62	0.05 ± 0.47	-0.18 ± 0.34	-0.33 ± 0.31	3.53 ± 4.45
	30	0.75 ± 0.74	0.57 ± 0.64	0.35 ± 0.58	0.02 ± 0.43	-0.22 ± 0.25	5.74 ± 7.63
	50	-0.07 ± 1.25	-0.1 ± 1.15 <sup>*</sup>	-0.02 ± 0.99	-0.45 ± 0.96	-0.62 ± 0.94	-0.35 ± 11.41

The increase in the body temperature was significantly reduced in the low-dose *V. yedoensis* BU fraction group at 0.5, 1.0, and 2.0 h after the LPS challenge ( $P < 0.01$  or 0.05). No significant difference was observed in the mid- and high-dose groups (Table 2).

In addition, the increase in the body temperature was significantly reduced in the aspirin group at 0.5 ( $P < 0.01$ ) and 1.0 h ( $P < 0.01$ ) after the LPS challenge. A reduction was also observed at subsequent time points, but the difference was not statistically significant (Table 2).

In conclusion, the *V. yedoensis* ethanolic extract and its fractions could reduce the body temperature with varying degrees of rabbits with LPS-induced fever, and their effects lasted longer than that of aspirin. The TRI results indicated that the active extract of *V. yedoensis* was the ethanolic extract and the strongest effect was produced by the EA and PE fractions, while the effect of BU fraction was relatively weak. It indicated that the antipyretic components of *V. yedoensis* were moderately polar or liposoluble.

### 3.3 Effects of each *V. yedoensis* extract on blood cells of febrile rabbits challenged with LPS for 6 h

Blood samples were collected from the rabbits in each group after the last temperature measurement (LPS challenge for 6 h) and examined by a hematology analyzer. The total WBC count ( $\times 10^3/\mu\text{L}$ ) in the model group ( $8.2 \pm 1.3$ ) was lower than and closer to that in the normal group ( $8.7 \pm 4.0$ ), and it was higher in each treatment group than that in the model group, except the water extract group. The above-mentioned finding indicated the effect of increasing WBC count (Figure 1). The PLT ( $\times 10^3/\mu\text{L}$ ) count in the model group ( $393.0 \pm 118.2$ ) was lower than that in the normal group ( $459.5 \pm 129.0$ ), and it was higher in the mid- and high-dose ethanolic extract groups, low- and mid-dose PE groups, high-dose EA group, and low- and mid-dose BU groups than that in the model group, thus indicating the inhibitory effect on PLT reduction (Figure 2). In terms of the WBC differential count, neutrophils (Neut) accounted for the largest portion of the WBCs. Moreover, Neut amount was higher in the model group ( $78.6 \pm 4.8\%$ ) than that in the normal group ( $69.2 \pm 10.1\%$ ); the Neut amount of all treatment groups, except the high-dose EA group and BU group, was lower than that in the

model group and close to that in the normal group (Figure 3). Lymphocytes (Lymph) amount was lower in the model group ( $14.2 \pm 1.7\%$ ) than that in the control group ( $23.9 \pm 9.3\%$ ), and the Lymph in each treatment group was higher than that in the model group and close to that in the normal group (Figure 4). None of the above-mentioned differences among the experimental groups were statistically significant.

### 3.4 Effects of each *V. yedoensis* extract on serum $\text{CH}_{50}$ level of rabbits with LPS-induced fever

Serum samples were collected from the rabbits in each group after the last temperature measurement (LPS challenge for 6 h), after which a microplate reader measured their  $\text{CH}_{50}$  levels. The results revealed that the  $\text{CH}_{50}$  level was higher in the model group ( $93.04 \pm 42.12$ ) than that in the normal group ( $69.10 \pm 22.31$ ), but it was lower in all the treatment groups, except the mid- and high-dose ethanolic extract groups than that in the model group. These findings indicated an inhibitory effect on an increase in the  $\text{CH}_{50}$  level. However, none of the difference among the experimental groups was statistically significant (Figure 5).

### 3.5 Overall results

The experimental results suggested that the PE and EA fractions of the ethanolic extract might be the main active *V. yedoensis* fractions with antipyretic effect. Moreover, their antipyretic effects lasted at least 3.0 h, significantly longer than those demonstrated in the aspirin group (1.5 h). In particular, the antipyretic effects lasted up to 4.0 h in the low- and mid-dose EA groups.

Although *V. yedoensis* had no significant effect on the blood cells of febrile rabbits challenged with LPS for 6.0 h, its ethanolic extract and PE fraction inhibited an inflammatory response and promoted coagulation.

In addition, although *V. yedoensis* had no significant effect on the serum  $\text{CH}_{50}$  levels of febrile rabbits challenged with LPS for 6.0 h, its water extract and different ethanolic extract fractions were shown to antagonize the activation of the complement system because of the LPS challenge and thus help to suppress an inflammatory response.

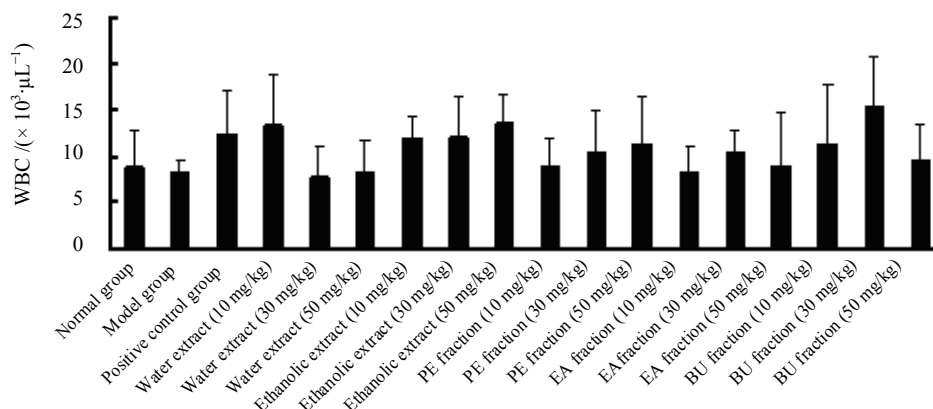


Figure 1 Effects of each *V. yedoensis* extract on WBC count of febrile rabbits challenged with LPS for 6.0 h ( $\bar{x} \pm s$ ,  $n = 6$ )

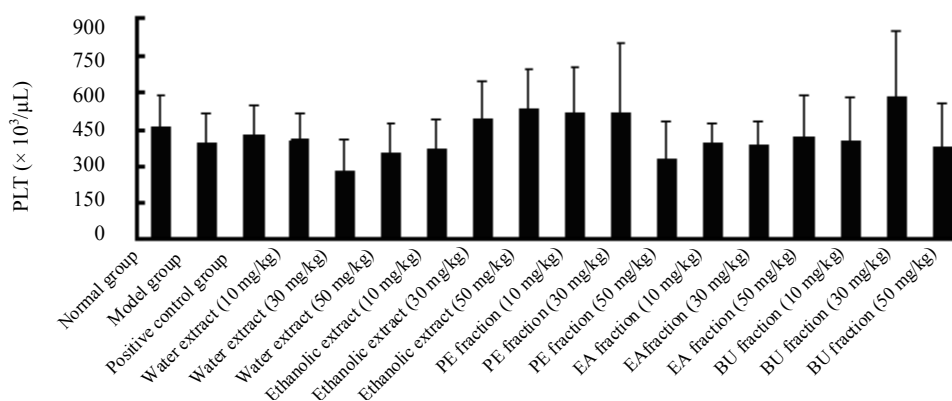


Figure 2 Effects of each *V. yedoensis* extract on PLT count of febrile rabbits challenged with LPS for 6.0 h ( $\bar{x} \pm s, n = 6$ )

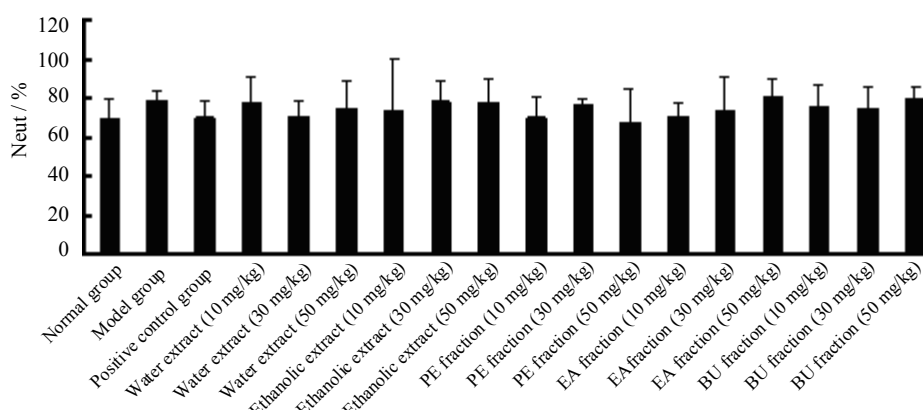


Figure 3 Effects of each *V. yedoensis* extract on Neut count of febrile rabbits challenged with LPS for 6.0 h ( $\bar{x} \pm s, n = 6$ )

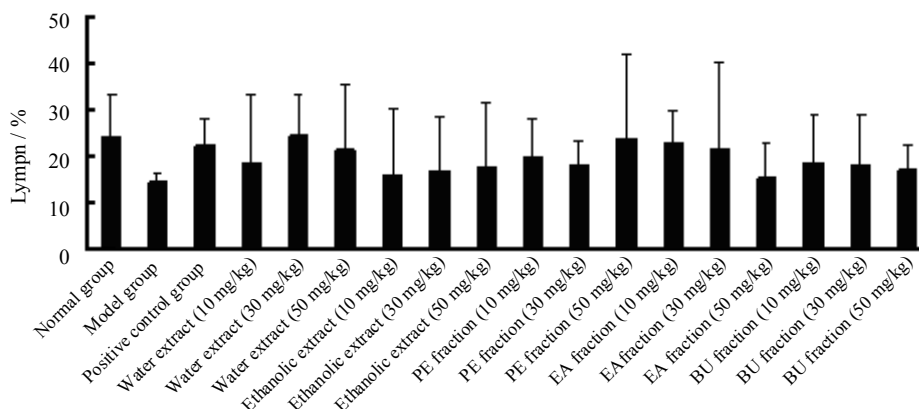


Figure 4 Effects of each *V. yedoensis* extract on Lymphn count of febrile rabbits challenged with LPS for 6.0 h ( $\bar{x} \pm s, n = 6$ )

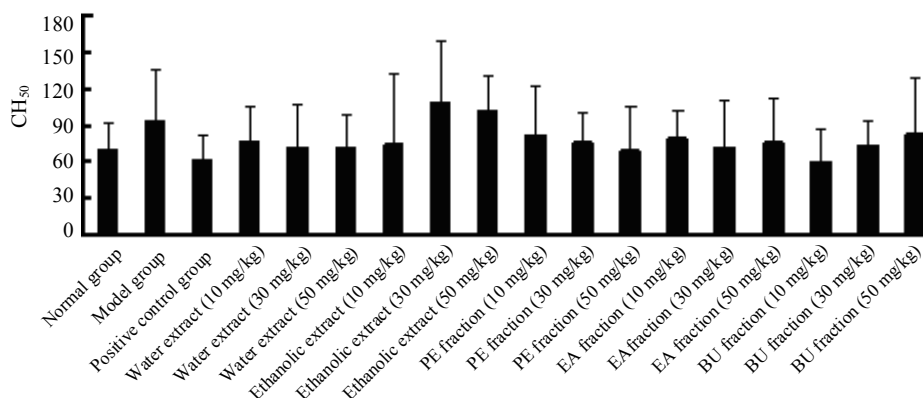


Figure 5 Effects of each *V. yedoensis* extract on serum CH<sub>50</sub> level of febrile rabbits challenged with LPS for 6.0 h ( $\bar{x} \pm s, n = 6$ )

#### 4. Discussion

Fever is an important sign of excess-heat syndrome. Many heat-clearing drugs or prescriptions have the obvious antipyretic effect, so an antipyretic experiment is a common and important indicator for investigating heat-clearing drugs. Bacterial endotoxin is exogenous pyrogen, which is mainly the LPS from the outer layer of cell wall of gram-negative bacteria. After entering the circulatory system of animal, pyrogen stimulates immune cells to release cytokines, i.e., endogenous pyrogen, which acts on the heat-regulating center and thus result in increased heat production and decreased heat dissipation, so the body temperature of animal increases. LPS has high pyrogenicity. Furthermore, iv injection of high-dose (2  $\mu\text{g}/\text{kg}$ ) LPS could cause two peaks in the temperature-time curve of rabbits that appeared at 1.5 h and 3.5 h, respectively (Kurland and Bockman, 1978). The LPS dose was 0.25  $\mu\text{g}/\text{kg}$  in the present experiment. However, the temperature-time curve of the model group also had two peaks, which appeared 0.5 and 3.0 h after the LPS challenge, respectively. After that, the increase in the body temperature was gradually reduced, and the temperature tended to be normal at 6.0 h. The EA and PE fractions of *V. yedoensis* ethanolic extract were shown to inhibit the increase in the body temperature of rabbits with varying degrees at each time point from 0.5 h to 4.0 h. Thus, it indicated the two fractions had a fast-acting and prolonged antipyretic effect. Moreover, to our knowledge, we presented the first evidence regarding the traditional heat-clearing effect of *V. yedoensis*.

Inflammation is a common and complex pathological process. LPS is a strong inflammatory stimulator that can activate inflammatory cells such as leukocytes and can initiate an inflammatory cascade, thus leading to tissue cell damage and organ dysfunction. Inflammation can cause the changes in the total WBC count and relative proportions of each WBC type. He et al (2011) observed a dynamic change over time in the total WBC count of endotoxemic rabbits after an LPS (10  $\mu\text{g}/\text{kg}$ ) challenge. With the LPS challenge, WBCs traversed out of the vessels and gathered in infected and injured fractions to play a key defensive role, so the total WBC count was reduced during the first period (0–2.0 h) after the LPS challenge. Subsequently, LPS would bind to its receptor to activate the production of a wide range of inflammatory mediators from the inflammatory cells that further activated inflammatory cells and promoted the release of WBCs from the bone marrow into the blood. As a result, the total WBC count gradually increased and was close to that in the normal group during the second (2.0–6.0 h) and third (6.0–36 h) periods. In addition, Neuts accounted for the largest portion of WBCs, and the Neut amount was higher in the treatment group than that in the blank control group. Invasion of LPS also activated the coagulation system, thus resulting in disseminated iv coagulation (DIC), which consumed many PLTs. Therefore, a progressive decrease in the PLT count was observed during the second and third periods.

Although the *V. yedoensis* extracts had no significant effect on the blood cells of febrile rabbits challenged with

LPS for 6.0 h in the present study, the responses in the normal and model groups coincided with the findings of He et al (2011). The total WBC count ( $\times 10^3/\mu\text{L}$ ) in the model group was lower than and close to that in the normal group, while an increase in the total WBC count was observed in the treatment groups with an ethanolic extract and its fractions. Neuts accounted for the highest proportion (78.6%) of the WBCs at that time point, and the Neut amount was higher in the model group than that in the normal group. Meanwhile, the Neut amount was decreased in the most treatment groups, which reduced the release of inflammatory mediators, thus producing an effect of inhibiting the inflammatory response. Besides, the effect of the PE fraction was relatively more significant. The PLT count ( $\times 10^3/\mu\text{L}$ ) was lower in the model group than that in the normal group, while the PLT count was higher in some treatment groups (e.g., the low- and mid-dose PE fraction groups and the mid- and high-dose ethanol extract groups) than that in the model group. Moreover, it demonstrated an antagonizing effect on an LPS-induced PLT reduction and thus promoted the coagulation in these treatment groups.

Some researchers demonstrated that after a 1–5  $\mu\text{g}/\text{kg}$  injection of LPS, rabbits would have a fever and relevant signs and symptoms that would generally return to normal within 8.0 h (He et al, 2011). In view of the findings of He et al (2011) the differences among each experimental group in the current study were not statistically significant in terms of the blood cells of rabbits with LPS-induced fever. It is possibly because the LPS doses were less than 1  $\mu\text{g}/\text{kg}$  and the blood cells were measured 6 h after the LPS challenge when the signs and symptoms of rabbits were returning to normal.

Complement is not only an important part of innate immune defense, but also one of the main mechanisms for antibody immune effects and has a regulatory role in the function of the immune system. Complement can be activated through a range of enzymatic cascade reactions. According to modern medicine, there are three complement activation pathways: (1) classical pathway-activation by antigen-antibody complexes, which require the involvement of antibodies to act on a bacterial infection (Colten and Rosen, 1992; Cooper, 1985; Ferguson et al, 2004; Neth et al, 2002; Villers et al, 2003); (2) alternative pathway-direct activation by pathogenic microorganisms; and (3) lectin pathway-activation by the mannose residues of pathogens that can be achieved even in the absence of an antibody (Muller-Eberhard and Schreiber, 1980; Farries and Atkinson, 1991; Reid and Turner, 1994; Winkelstein et al, 1976). In the present experiment, the total serum complement  $\text{CH}_{50}$  levels of the rabbits were measured by hemolysis, which mainly reflected the classical complement activation pathway.

When rabbits were challenged with LPS, the complement system was activated by an infection, and the total serum complement level was increased. The results revealed that the total serum complement level was higher in the model group than that in the normal group. Besides, the complement system produced inflammatory factors, indirectly

released inflammatory mediators and cytokines, and thus participated in the inflammatory response. On the other hand, the complement system expanded and intensified the inflammatory response through the interaction with the coagulation system, thus involving the pathophysiology of a variety of infectious and non-infectious inflammatory diseases. Therefore, timely and appropriate suppression of excessive activation of the complement system is an effective way of treating inflammatory diseases. In this experiment, the  $CH_{50}$  levels were lower in the groups treated with *V. yedoensis* water extract and various fractions in ethanolic extract than that in the model group ( $P > 0.05$ ). It indicated that some *V. yedoensis* extracts had an effect of antagonizing the increase of  $CH_{50}$  and thus helped to suppress the inflammatory response. The above-mentioned experimental results are consistent with the results of our recent study that could indicate an anti-complement activity of the PE fraction of *V. yedoensis* *in vitro* could inhibit the acute lung injury in mice. In addition, we speculate that the heat-clearing drug, *Viola Herba*, may treat the acute lung injury through its antipyretic effect and inhibition of an inflammatory response.

## 5. Conclusion

The results obtained in this study suggest that the PE and EA fractions of the ethanolic extract from *V. yedoensis* show significantly antipyretic effect on the LPS-induced rabbit fever. These data also suggest that the liposoluble fraction of *V. yedoensis* promise as a new effective antipyretic agent. To our knowledge, the antipyretic effect contributes partly to the heat-clearing function of the Chinese materia medica.

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