Tropical Journal of Pharmaceutical Research October 2017; 16 (10): 2395-2401
**ISSN:** 1596-5996 (print); 1596-9827 (electronic)


Available online at http://www.tjpr.org
**http://dx.doi.org/10.4314/tjpr.v16i10.12**

**Original Research Article**

# Effects of flavonoids extracted from the whole plant of Patrinia Villosa (Thunb) Juss in a rat model of chronic pelvic inflammation

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*Sent for review: 3 June 2017* *Revised accepted: 11 September 2017*

## Abstract

***Purpose:*** *To investigate the effects of total flavonoids (PLV) extracted from the whole plant of Patrinia Villosa (Thunb.) Juss (PTJ) in a rat model of chronic pelvic inflammation.*
***Methods:*** *An orthogonal test design was employed to optimize the extraction conditions of PLV via reflux extraction by ethanol. Rats were randomly divided into control group, model group and PLV groups. An absorbable gelatin sponge with pathogens was inserted into the cervix of the rat to establish a pelvic inflammatory model. The PLV groups were orally administered PLV at doses of 25, 50 and 100 mg/kg for eight days. Enzyme-linked immunosorbent assay (ELISA) was used for the determination of inflammatory cytokines in rat serum and the culture supernatant of RAW264.7 cells. Real-time reverse transcription polymerase chain reaction (real-time PCR) was employed to determine mRNA levels.*
***Results:*** *The optimum extraction conditions for PLV by orthogonal test were obtained: extraction time (120 min), ratio of liquid to raw material (20 mL/g) and ethanol concentration (50 %). By treating with PLV, the levels of TNF-α, IL-6 and IL-1β significantly decreased ($p < 0.01$), while IL-10 level significantly increased ($p < 0.01$) in the serum of chronic pelvic inflammatory rats and LPS-stimulated macrophages. In addition, a similar trend was observed in the mRNA levels of LPS-stimulated macrophages treated with PLV.*
***Conclusion:*** *PLV showes significant anti-inflammatory effects on chronic pelvic inflammation. The potential mechanism is related to regulating the expression of inflammatory factors*

***Keywords:*** *Patrinia Villosa (Thunb.) Juss, Total flavonoids, Chronic pelvic inflammation, Inflammatory cytokines*

**Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts**

## INTRODUCTION

Pelvic inflammatory disease (PID) is a common gynecological disease which seriously affects the life quality of woman, and it usually causes chronic pelvic pain, tubal factor infertility, and ectopic pregnancy. The etiopathogenisis of PID is the infection of pathogenic microorganisms in upper genital tract which causes endometritis, salpingitis, and peritonitis, etc. [1,2]. *Chlamydia trachomatis* and *Neisseria gonorrhoeae* were proved to be the most common pathogen causes of PID [3]. Antibiotics are usually used for the treatment of PID to reduce short-term morbidity. However, they have no effects on long term complications induced by PID [4]. In addition, bacterial drug resistance and drug side effects limit the clinical use of antibiotics [5]. Therefore,

new drugs are needed for inhibiting the progression and alleviating the long-term sequelae of PID.

*Patrinia villosa (Thunb.)* Juss. (PTJ), called *Baijiangcao* in Chinese, is an important herbal medicine which has been used in China for more than 2000 years. It belongs to the family of Valerianaceae, and has been used in Traditional Chinese Medicine (TCM) for treating inflammation, wound and abdominal pain [6]. Previous reports have revealed that triterpenoid saponins, iridoids, flavonoids, flavonones, and polysaccharides are the major bioactive constituents in PTJ, which displayed anti-tumor and anti-inflammatory activities [7]. However, to the best of our knowledge, there is no report regarding the effects of flavonoids from PTJ on chronic pelvic inflammatory disease. In the present study, orthogonal design was used to optimize the extraction conditions of flavonoids from PTJ, and their effects on chronic pelvic inflammation were studied *in vivo* and *in vitro*.

## EXPERIMENTAL

### Chemicals and reagents

Rutin standard was obtained from National Institute for Food and Drug Control (Beijing, China). ELISA kits for interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6 and IL-10 were obtained from R&D Systems (Minneapolis, MN, USA). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo (Kumamoto, Japan). Absorbable gelatin sponge was purchased from Jinling Pharmaceutical Co. (Nanjing, China). The herbs of PTJ was purchased from an herbal medicine market (Ji'nan, China), and authenticated by a taxonomist in the Department of Traditional Chinese Medicine in Yuhuangding Hospital of Yantai (Yantai, China). A voucher specimen (no. CYC-20160803) was deposited in the herbarium of Yuhuangding Hospital of Yantai (Yantai, China).

### Extraction of flavonoids (FLV) from PTJ

PTJ was dried in a drying oven at 50 °C and 10 g powder was extracted by reflux with designed extraction conditions (extraction time, ratio of liquid to raw material and ethanol concentration) (Table 1). The solutions were collected and cooled to room temperature. After centrifuging at 3000 rpm for 10 mins, the supernatants were collected and filtered through a nylon filter (0.22 μm). The determination of flavonoids was performed by using the aluminum nitrate colorimetric method at 500 nm [8]. Rutin was used as the standard to calculate the total flavonoid content.

**Table 1:** Factors and their levels used in the orthogonal design

| Factor | Level | | |
|---|---|---|---|
| | ***1*** | ***2*** | ***3*** |
| A: Extraction time (min) | 60 | 120 | 180 |
| B: Ratio of liquid to raw material (mL/g) | 10 | 20 | 30 |
| C: Ethanol concentration (%) | 40 | 50 | 60 |

### Optimization of flavonoid extraction

In the present study, an orthogonal test ($L_9(3^4)$) was used to investigate the effects of extraction time (min), ratio of liquid to raw material (mL/g), and ethanol concentration (%) on the extraction yield of PLV. The factors and experimental data are shown in Table 1 and Table 2.

### Animals

Female Sprague-Dawley (SD) rats (aged 12 weeks, 190 ± 10 g) were obtained from Laboratory Animal Center of Shandong University (Jinan, China). All rats were housed in an controlled environment (21 ± 2 °C, 55 ± 5 % humidity) with a 12 h light/12 h dark cycle. All procedures of animal experiments in the present study were approved by Institutional Animal Care and Use Committee at Yuhuangding Hospital of Yantai (approval no. 201609-1L) and in accordance with "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [9].

### Cell culture

Murine macrophage RAW 264.7 cells was obtained from the American Type Culture Collection (ATCC, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum (FBS) and antibiotics (100 mg/mL streptomycin and 100 U/mL penicillin), and cultured in 5 % $CO_2$/95 % air at 37 °C.

### Determination of anti-inflammatory effect of PLV

After acclimation for 7 days, the rats were injected progesterone (10 mg) subcutaneously. The PID model was constructed using the previous method with some modifications [10]. An absorbable gelatin sponge (0.125 $mm^3$) was immersed in the mixed pathogen solution (1 × $10^8$ ccu/mL *U. urealyticum* (*t*-strain mycoplasma) and 1 × $10^8$ cfu/mL pathogenic *E. coli*). Then the

gelatin sponge was inserted into the cervix of each rat and the rat was forced upside down for 5 min. A gelatin sponge without microbe was inserted into the cervix of each rat in control group. Infections were performed for four times with a 2-day interval. PLV were orally administrated at the doses of 25, 50 and 100 mg/kg for eight days. Thereafter, the rats were anaesthetized by injecting pentobarbital (30 mg/kg) subcutaneously and blood was collected from the abdominal aorta to obtain serum.

### Cell viability assay

Cell viability of RAW 264.7 cells was evaluated using a Cell Counting Kit-8 (CCK-8). Cells ($1 \times 10^4$ cells/well) were transplanted into a 96-well microplate and incubated for 24 h at 37 °C. PLV was added to each well to make the final concentrations of 25, 50, 100, 200, 400 and 800 μg/mL, and incubated for another 24 h. CCK-8 reagent was added to each well and incubated for 4 h. Absorbance was read at 450 nm using a microplate reader. Cell viability was calculated using Eq 1.

$$\text{Cell viability (\%)} = (A_1/A_0)100 \quad \text{..... (1)}$$

where $A_1$ is the absorbance of PLV treated cells, and $A_0$ the absorbance of PLV untreated cells.

### Determination of inflammatory cytokines by ELISA

Cells were treated with PLV (50, 100 and 200μg/mL) for 4 h, and then LPS (1 μg/mL) was added and incubated for another 24 h. Levels of IL-6, IL-1β, IL-10 and TNF-α in the supernatants of cell culture were measured using commercial ELISA kits following the manufacturer's instructions.

### Real-time reverse transcription polymerase chain reaction (real-time PCR)

A fluorescence quantitative Light Cycler 480 Real Time PCR system (Roche, Sweden) was used to determine the mRNA expressions of TNF-α, IL-6, IL-1β, and IL-10. Total RNA was extracted from the cells by Trizol reagent and equal amounts of RNA was reverse transcribed to cDNA using SYBR Green qPCR SuperMix (Invitrogen) following the manufacturer's protocol. The PCR oligonucleotide primers are shown in Table 2. The ΔΔCt method was used to calculate the relative gene expression.

### Statistical analysis

Statistical analysis was carried out using SPSS software 18.0 for Windows (SPSS Inc., Chicago, IL, USA). All data are presented as mean ± standard deviation (SD) and comparison between two groups was performed by Student's *t* test. $P < 0.05$ was considered statistically significant.

## RESULTS

### Optimized extraction conditions

The experimental results from orthogonal test were shown in Table 3. The factors were quantitatively analyzed using evaluation indices K and R. It is known that the factor with the larger R value have the greater effect on the extraction yield. Thus, the influential order of the three factors was A > B > C. Furthermore, variance analyses (ANOVA) in Table 4 shows that the effects of three tested factors ($p < 0.01$, $p < 0.05$ and $p < 0.05$ for A, B and C, respectively) on extraction yield were significant. Based on the R values and ANOVA, the optimum extraction conditions for PLV were: extraction time (120 min), ratio of liquid to raw material (20 mL/g) and ethanol concentration (50 %). A validation experiment were carried out using the optimum extraction conditions, and resulted in a total yield of 43.34 ± 1.23 mg/g.

### Effect of PLV on inflammatory cytokines in rat serum

The results are shown in Figure 1. After model construction, the serum levels of IL-6, IL-1β and TNF-α significantly increased ($p < 0.01$, compared with the control group).

**Table 2:** Oligonucleotide primers for real-time RT-PCR analysis

| Target gene | Primer sequence |
|---|---|
| TNF-α | F: 5′-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3′<br>R: 5′-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3′ |
| IL-6 | F: 5′-TCCAGTTGCCTTCTTGGGAC-3′<br>R: 5′-GTGTAATTAAGCCTCCGACTTG-3′ |
| IL-1β | F: 5′-ATGGCAACTGTTCCTGAACTCAACT-3′<br>R: 5′-CAGGACAGGTATAGATTCTTTCCTTT-3′ |
| IL-10 | F: 5′-TCT ACA AGGCCA TGA ATG AG-3′<br>R: 5′-GAG AGA GGTACA AAC GAG G-3′ |

**Table 3:** Orthogonal design and experimental results

| No. | A | B | C | D | Yield (mg/g) |
|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 1 | 25.1 |
| 2 | 1 | 2 | 2 | 2 | 37.2 |
| 3 | 1 | 3 | 3 | 3 | 35.3 |
| 4 | 2 | 1 | 2 | 3 | 38.5 |
| 5 | 2 | 2 | 3 | 1 | 42.5 |
| 6 | 2 | 3 | 1 | 2 | 37.5 |
| 7 | 3 | 1 | 3 | 2 | 26.6 |
| 8 | 3 | 2 | 1 | 3 | 28.8 |
| 9 | 3 | 3 | 2 | 1 | 32.7 |
| K1 | 32.533 | 30.067 | 30.467 | 33.433 | |
| K2 | 39.500 | 36.167 | 36.133 | 33.767 | |
| K3 | 29.367 | 35.167 | 34.800 | 34.200 | |
| R | 10.133 | 6.100 | 5.666 | 0.767 | |

**Table 4:** Analysis of variance of the studied factors

| Source | Sum of squares | Degree of freedom | *F*-ratio | $F_{0.05}/F_{0.01}$ | Significant |
|---|---|---|---|---|---|
| A | 161.247 | 2 | 181.789 | 99.00 | ** |
| B | 64.220 | 2 | 72.401 | 19.00 | * |
| C | 52.667 | 2 | 59.377 | 19.00 | * |
| D | 0.887 | 2 | 1.000 | 19.00 | |
| Error | 0.89 | 2 | | | |

Compared with the rats in the model group, serum levels of the three inflammatory factors significantly decreased ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively) by treating with PLV at the concentrations of 25, 50 and 100 mg/kg. However, the serum level of IL-10 in PLV treated rats significantly increased when compared with model rats at the concentrations of 50 and 100 mg/kg ($p < 0.01$).

## Effect of PLV on RAW264.7 cell viability

The effects of PLV on RAW264.7 cell viability were evaluated by MTT assay. As can be seen from Figure 2, no significant effect was observed on the viability of RAW264.7 cells by treating PLV at concentrations ranged from 25 to 800 μg/mL. As a result, the concentrations of 50, 100 and 200 μg/mL were selected for further experiments.

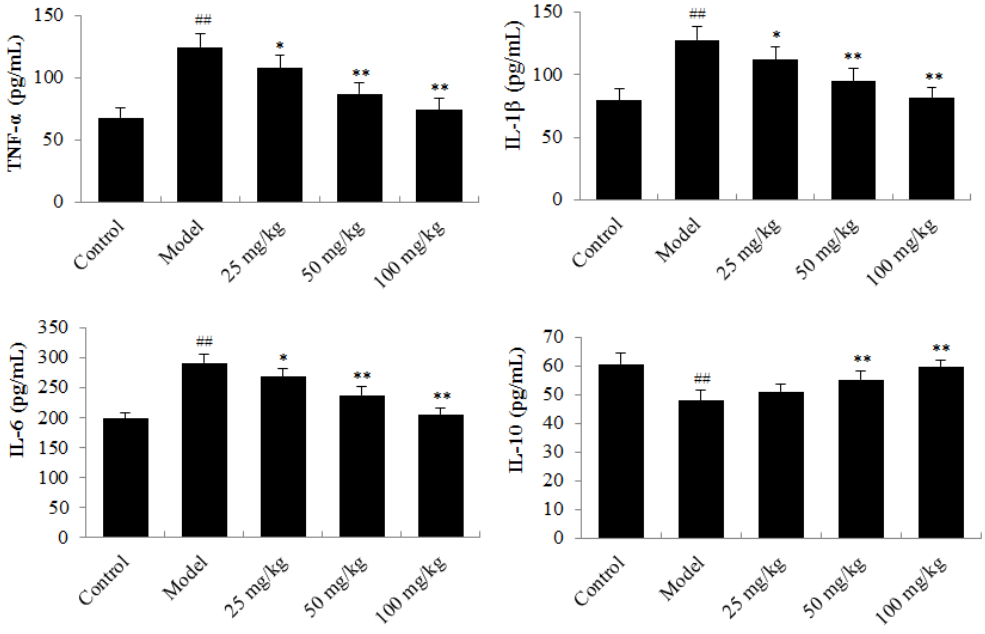


**Figure 1:** Effects of PLV on levels of TNF-α, IL-6, IL-1β and IL-10 in the serum of rats

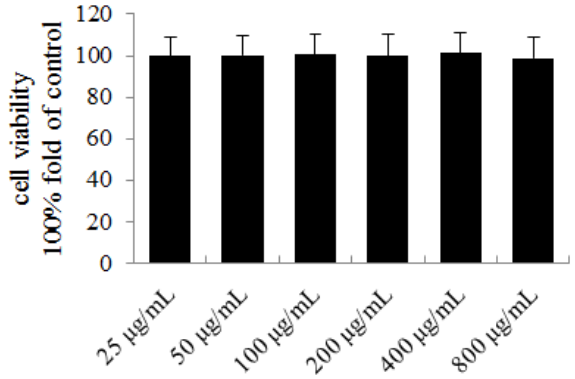


**Figure 2:** Effect of PLV on cell viability of RAW264.7 cells

### Effects of PLV on inflammatory cytokines *in vitro*

Effects of PLV on the levels of cytokines (TNF-α, IL-1β, IL-6 and IL-10) in RAW264.7 cells were determined by ELISA. As shown in Figure 3, LPS stimulation significantly increased the levels of the cytokines ($p < 0.01$). Compared with LPS-stimulated model cells, the levels of TNF-α, IL-6, IL-1β significantly decreased, whereas the level of IL-10 significantly increased in PLV-treated (50, 100 and 200 μg/mL) cells with a concentration-dependent manner.

### Effect of PLA on expression of mRNA of cytokines

To further investigate the anti-inflammatory mechanism of PLV, real-time RT-PCR was employed to investigate the mRNA levels of inflammatory cytokines. As shown in Figure 4, LPS treatment significantly increased the mRNA expression of the cytokines ($p < 0.01$). The mRNA expression of cytokines (TNF-α, IL-6 and IL-1β) were significantly down-regulated by treatment of PLA (50, 100 and 200 μg/mL). The mRNA expression of IL-10 was significantly up-regulated by treating PLV (100 and 200 μg/mL).

## DISCUSSION

It is reported that traditional Chinese medicines (TCM) may provide a promising strategy to prevent chronic inflammatory diseases [11,12]. Flavonoids have been isolated and characterized from a variety of plants [13,14], and they are also dominant bioactive constituents in PTJ [6,7]. In the present study, flavonoids were extracted from PTJ and the extraction conditions were optimized by employing an orthogonal design. A total PLV yield of 43.34 ± 1.23 mg/g was obtained by validation experiments and PLV was proved to have obvious anti-inflammatory effects on chronic pelvic inflammatory disease by *in vivo* and *in vitro* experiments.

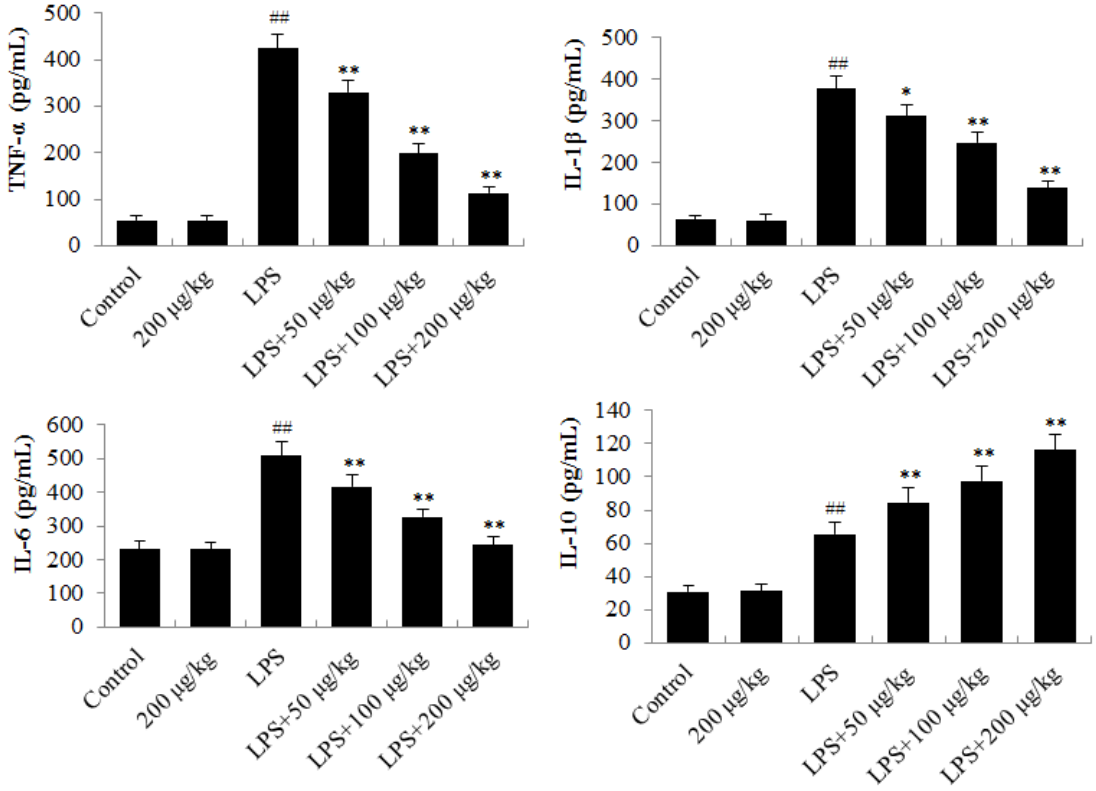


**Figure 3:** Effects of PLV on the levels of cytokines in RAW264.7 cells

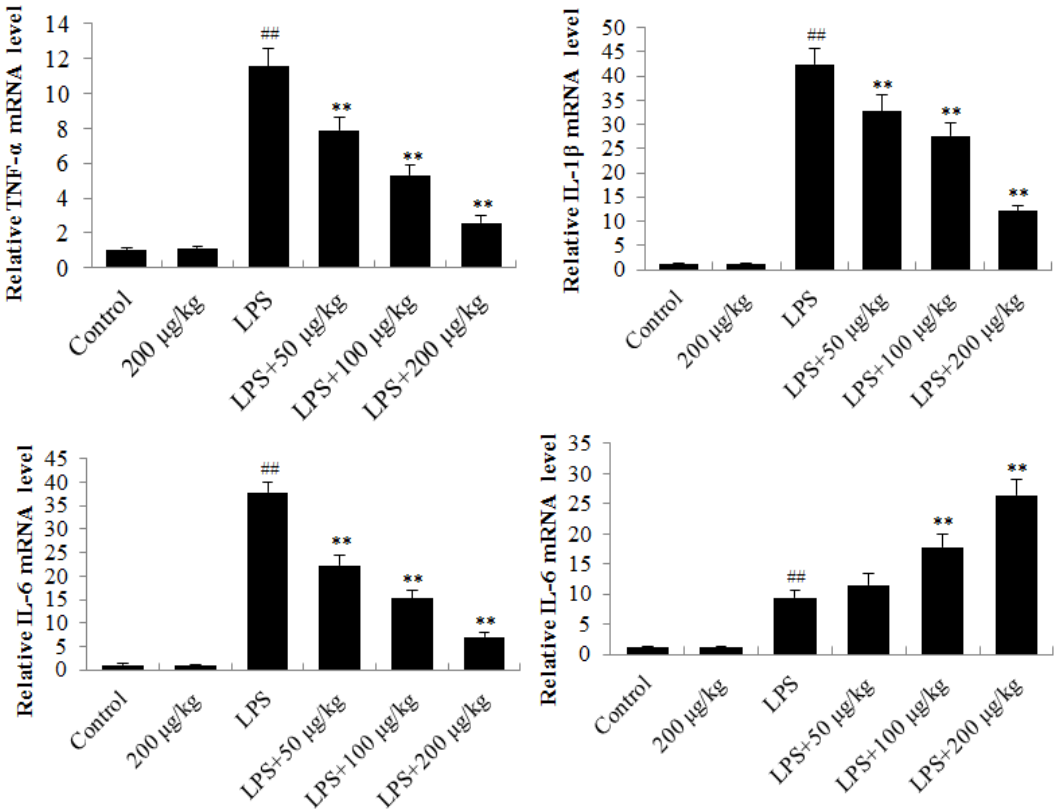


**Figure 4:** Effect of PLA on mRNA expression TNF-α, IL-6, IL-1β, and IL-10 in RAW264.7 cells

Inflammatory response in upper genital tract can be initiated and propagated by the overproduction of pro-inflammatory cytokines, which can also activate local immune cells and increase chemokines productions. Chemokines promote the infiltrations of hematopoietic immune cells, and then the productions of pro-inflammatory cytokines are further increased and the inflammatory response intensified [15,16]. Hence, inflammatory cytokines play a central role in inflammatory response. In the present study, the effects of PLV on serum levels of inflammatory cytokines were evaluated in the PID rats. The results indicated that PLV decreased the serum levels of TNF-α, IL-6 and IL-1β, while increased the IL-10 level in the rats.

A large number of studies have demonstrated that overproduction of different pro-inflammatory mediators and cytokines by macrophages may be the major factor in the process of inflammatory responses [17,18]. In the present study, the RAW264.7 cells were used to evaluate the effects of PLV on inflammatory cytokines. The results showed that treatment of PLV significantly suppressed the levels of TNF-α, IL-1β, IL-6, whereas increased the IL-10 level in LPS-stimulated RAW264.7 cells. In addition, the results of mRNA expression were consistent with the results of inflammatory cytokines production.

## CONCLUSION

The findings of the present study indicate that PLV possesses significant anti- inflammatory effects on chronic pelvic inflammation, and that the mechanism is closely related to a decrease in the levels of TNF-α, IL-1β and IL-6, as well as an increase in IL-10 level. Thus, PLV has potentials to be developed into anti-inflammatory drugs for chronic pelvic inflammation in the future.

## DECLARATIONS

### *Acknowledgement*

None.

### *Conflict of Interest*

No conflict of interest associated with this work.

### *Contribution of Authors*

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

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