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Pulsatilla decoction inhibits *Candida albicans* proliferation and adhesion in a mouse model of vulvovaginal candidiasis via the Dectin-1 signaling pathway

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ABSTRACT

Ethnopharmacological relevance: Pulsatilla decoction (PD) is a classical prescription in Traditional Chinese Medicine (TCM) and has been reported to have inhibitory effects on *Candida albicans* proliferation.
Study aim: To investigate the therapeutic effects of PD in the treatment of Vulvovaginal candidiasis (VVC) and elucidate the potential mechanism.
Materials and methods: Female BALB/c mice (N = 90) were randomized to six treatment groups, including the Control group, Model group, three PD groups and Fluconazole group which served as a positive control (20 mg/kg weekly). The three PD groups (low dose group, medium dose group and high dose group) were given a daily intragastric gavage of PD at doses of 5, 10 and 20 g/kg, respectively. Five animals from each group were euthanized on Day 4, Day 7 and Day 14 after treatment. Colony forming unit (CFU) was measured by the serial dilution method. The degree of infection was assessed by Gram staining, Periodic acid schiff (PAS) staining, Hematoxylin and eosin (H&E) staining and Scanning electron microscopy (SEM). The serum inflammation levels were determined by a Luminex assay. Gene and protein expression levels of components of the Dectin-1 signaling pathway were determined by Real-time PCR, Western-blot and immunohistochemistry, respectively.
Results: The administration of PD significantly decreased the fungal load from Day 7 post-infection onwards and decreased the number of visible microorganisms based on findings from Gram staining, PAS staining and SEM. H&E staining indicated that the impaired histological profiles were improved in all three PD groups. PD led to a significantly lower level of IL-23 in the serum; the levels of IL-10 and TNF-α were also decreased, although the differences were not significant. Furthermore, a substantial downregulation of Dectin-1, CARD9 and NF-κB mRNA levels and Dectin-1, Syk, CARD9 and NF-κB protein levels was observed after the administration of PD.
Conclusion: This study suggests that PD exerts inhibitory effects on *C. albicans* proliferation, adhesion and inflammation and simultaneously downregulates the expression levels of important genes and proteins associated with the Dectin-1 pathway, highlighting the potential application of PD to improve the clinical management of VVC.

1. Introduction

Vulvovaginal candidiasis (VVC) is one of the most common types of vaginitis and affecting 70–75% of women at least once in their lifetime, with 40–45% of women experiencing recurrence (Workowski and Berman, 2006). VVC is caused by *Candida* in its hyphal phase, and the clinical symptoms of VVC include obvious pruritus and burning pain in the vulvaes. In some severe cases, the patients may experience extreme pining and piercing pain. VVC is also closely associated with dyspareunia, dysuria and frequency of urination. As a result, VVC can

Abbreviations: PD, Pulsatilla decoction; TCM, Traditional Chinese Medicine; VVC, Vulvovaginal candidiasis; HPLC, High performance liquid chromatography; CFU, Colony forming unit; PAS, Periodic acid schiff; H&E, Hematoxylin and eosin; SEM, Scanning electron microscopy; CLR, C-type lectin receptor; PRR, Pattern recognition receptor; PAMP, Pathogen-associated molecular pattern; ITAM, Immunoreceptor tyrosine activation motif; Syk, Spleen tyrosine kinase; CARD9, Caspase recruitment domain-9; SDA, Sabouraud dextrose agar; ATCC, American Type Culture Collection; PBS, Phosphate buffered saline; RT-PCR, Real-time polymerase chain reaction; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, Polyvinylidene difluoride; EDTA, Ethylenediamine tetraacetic acid

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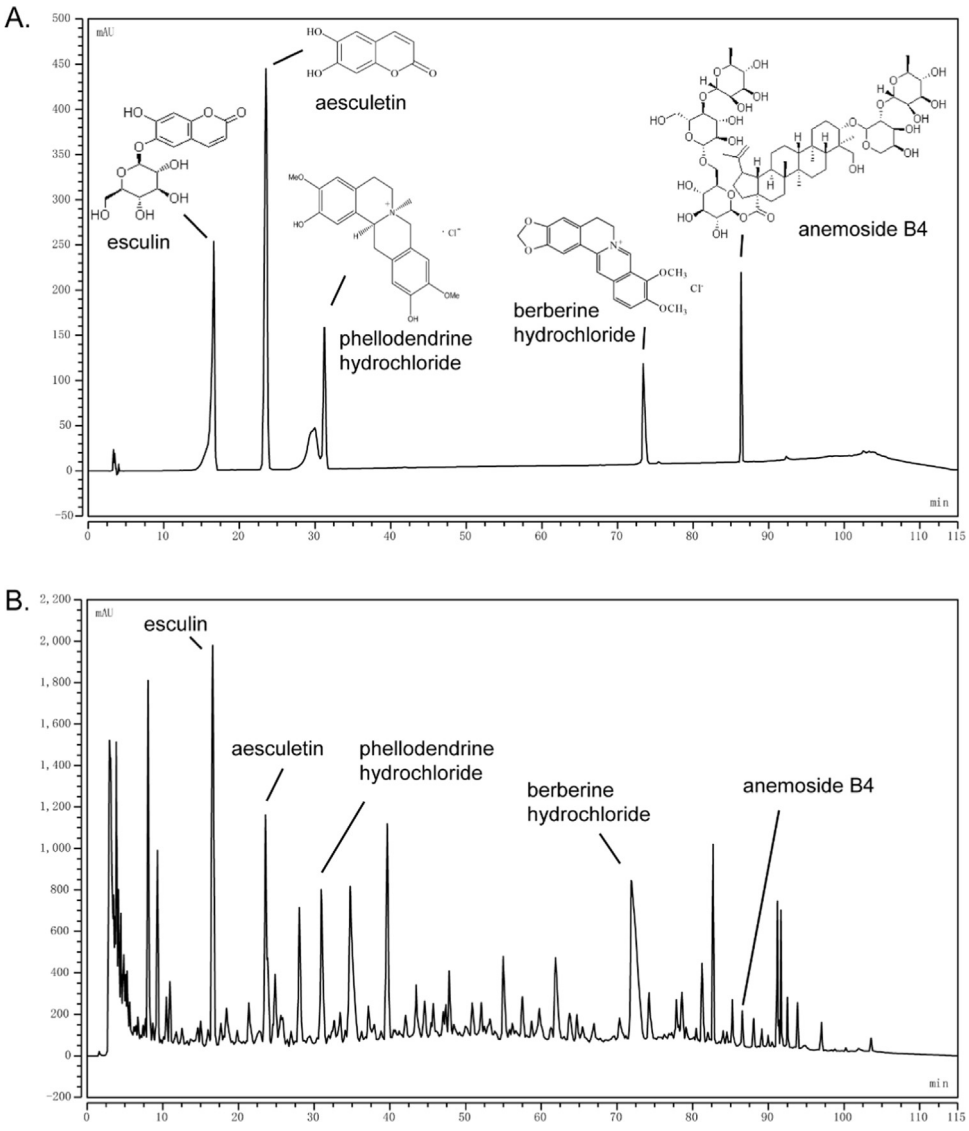

Fig. 1. HPLC analysis of the PD decoction extract. A. HPLC of the standard compounds. B. HPLC of the test solution.

markedly compromise quality of life in affected females (Cassone and Sobel, 2016).

Dectin-1 is a transmembrane protein and a member of the C-type lectin receptor (CLR) family. Accumulating evidence indicates that Dectin-1 serves as a pattern recognition receptor (PRR) and plays an essential role in defense against fungal infection (Drummond and Brown, 2011; Kerrigan and Brown, 2010). The pathogen-associated molecular pattern (PAMP) identified for Dectin-1 is β-glucan, a component of the fungal cell wall (Reid et al., 2009). At the onset of *Candida* invasion, the PRR (Dectin-1) recognizes β-glucan and triggers intracellular signaling, with phosphorylation of immunoreceptor tyrosine activation motif (ITAM). This phosphorylation provides a docking site for spleen tyrosine kinase (Syk), which plays a pivotal role in mediating the downstream cellular response (Reid et al., 2009). Syk recruits caspase recruitment domain-9 (CARD9) to activate downstream signaling components. Subsequently, CARD9 activates the canonical NF-κB signaling pathway, resulting in the production of inflammatory cytokines and chemokines (Li et al., 2009).

Currently, azole antifungal agents are the most commonly used therapeutic regimens for VVC. However, drug resistance emerges for conventional therapies due to overprescription (Coleman et al., 2015). In addition, adverse effects related to antifungal drugs such as gastrointestinal discomfort, rash and hepatotoxicity remain barriers for wider application. Hence, novel therapeutic approaches for VVC are needed for improved clinical management.

Over centuries of empirical clinical practice, compounds derived from Traditional Chinese Medicine (TCM) have been demonstrated to exert therapeutic effects by combining multiple components rather than targeting a single molecule (Sucher, 2013). An increasing number of studies suggested that TCM can serve as a rich resource of novel therapies. *Pulsatilla decoction* (PD), was first prescribed by Zhang Zhongjing in “Shang Han Lun”, approximately 1800 years ago. It is composed of 4 commonly used plants: *Radix pulsatilla* (Bai Tou Weng), *Cortex phellodendri* (Huang Bai), *Rhizoma coptidis* (Huang Lian) and *Cortex frax*ini (Qin Pi). The active ingredients from three of these four herbal plants (Bai Tou Weng, Huang Bai and Qin Pi) have been shown to exert anti-fungi effects in *vitro*(Liu et al., 2012; Yang et al., 2015). TCM theorizes that VVC is mainly caused by excess of damp-heat and PD is effective in correcting the dysregulation of heat and dampness. Therefore, PD is widely used in China to treat VVC and has shown satisfactory effects in clinical practice (Zhang and Zhang, 2012). While in *vitro*, studies indicated that PD has significant anti-*C. albicans* activity (Zhang et al., 2015a, 2015b), only few studies addressed the therapeutic effects of PD against *C. albicans in vivo*. In this study, we investigated the anti-*C. albicans* activity effects of PD at different doses in a mouse model of VVC. We further correlated the therapeutic effects of

PD with the Dectin-1 signaling pathway to support the use of PD as a promising therapeutic strategy for treating VVC in future clinical practice.

2. Materials and method

2.1. Preparation of PD

The PD consistes of *Pulsatilla chinensis* (Bunge) Regel.,Radix (15 g), *Phellodendron chinense* C.K. Schneid.,Cortex (12 g), *Coptis chinensis* Franch.,Rhizome (6 g), *Fraxinus rhynchophylla* Hence.,Cortex (12 g) (composition ratio = 5:4:2:4). All ingredients were purchased from the First Affiliated Hospital of Guangzhou University of Chinese Medicine. The ingredients were weighed and immersed in distilled water at an 8:1 vol/weight ratio for 30 min and boiled for 45 min. The supernatant was gauze filtered and concentrated by a rotary evaporator (70 °C) to a concentration of 2 g/ml and stored at 4 °C for further use.

2.2. Analysis of PD by high performance liquid chromatography (HPLC)

The PD fingerprint was investigated by using HPLC (Thermo Fisher, UltiMate™ 3000, USA). A Hypersil gold C_{18} column (250 mm × 4.6 mm, 5 μm; Thermo Fisher, Part no. 25005–254630, USA) was used with the column temperature set at 30 °C. The mobile phase consisted of acetonitrile (A) and 0.1% orthophosphoric acid (B). The gradient elution was performed as follows: 5% A from 0 to 5 min, 5–10% A from 5 to 30 min, 10–15% A from 30 to 45 min, 15–20% A from 45 to 65 min, 20–30% A from 65 to 85 min, 30–50% A from 85 to 100 min, 50–5% A from 100 to 110 min, with equilibration of the gradient elution for 5 min. The injection volume was 10 μl, and the analysis was performed at a flow rate of 1.0 ml/min with detection at 201 nm. PD was prepared as described above. Five reference substances were used for the qualitative analysis: esculin, aesculetin, phellodendrine hydrochloride, berberine hydrochloride and anemoside B4. The compounds were verified by comparing the individual peak retention times with those of the reference substances (Fig. 1).

2.3. Strains

The strain *C. albicans* ATCC10231 was obtained from the American Type Culture Collection (ATCC; USA). The strain was recovered and cultivated to the third generation in Sabouraud dextrose agar (SDA) at 35 °C for 72 h. The colonies were harvested using a laboratory coating rod and resuspended in sterile phosphate buffered saline (PBS). The final cell concentration was adjusted to 1×10^6 colony forming unit (CFU)/ml by using a Maxwell turbidimetric meter (Yue Fung, SGZ-2BXJ, China).

2.4. Animals

Eight–week-old female BALB/c mice (weight, 18–22 g) were purchased from the Medical Experiment Center of Guangzhou University of Chinese Medicine [licence number: SCXK (Yue): 2013–0034]. The mice were maintained in a 12 h/12 h light/dark cycle under regulated temperature (22 ± 2 °C) and humidity (50 ± 10%) and fed a standard diet and water ad libitum in the Biosafety Laboratory Level 2 (BSL2) of South China Agricultural University [licence number: SYXK (Yue): 2014–0136]. All protocols were reviewed and approved by the Institutional Animal Care and Ethics Committee of Guangzhou University of Chinese Medicine.

2.5. Groups and pharmacological administration

After adapting for 1 week, a total of 90 mice were equally randomized to 6 groups: the Control group, Model group, three PD groups (low dose group, medium dose group and high dose group) and Fluconazole group. All groups were established VVC model except for the Control group which served as a negative control. As previously described (Yano and Jr, 2011), pseudo-estrus was induced during infection by injecting subcutaneously 0.1 ml of sesame oil containing 0.2 mg of estradiol benzoate (Shusheng, China) in the lower abdominal area of the animals 3 days before inoculation (-Day 3). The estradiol treatments were repeated one day before infection (-Day 1). Infection of the vaginal canal was performed by inoculating the mice intravaginally with 20 μl of an inoculum suspension with a concentration of 1×10^6 CFU/ml on the next day (Day 0).

On Day 1 post-inoculation, the three PD groups were administered 2 ml of PD daily at doses of 5, 10 and 20 g/kg, respectively. The Fluconazole group was treated with fluconazole (SHIMEN, 1.3.1, China) at a dose of 20 mg/kg of body weight in 2 ml weekly. The Control group and the Model group received an equivalent amount of normal saline lavage. The general health status of the mice was monitored and weight was recorded every day. On Day 4, Day 7 and Day 14 after infection, vaginal lavage was collected for colony counts and Gram staining. Five animals were euthanized at each time point for blood and vaginal tissues collection. The blood was allowed to coagulate for approximately 1 h at room temperature and serum was obtained by centrifugation at 3000 rpm/min for 15 min and stored at − 80 °C for future use. The vaginal tissues were dissected into parts for fixation in 10% paraformaldehyde and 2.5% glutaraldehyde for histopathology and scanning electron microscopy (SEM), respectively. The tissues were also snap frozen and preserved at − 80 °C for mRNA and protein expression analysis.

2.6. Colony counts

Lavage fluid from the vaginal lumen was obtained by injecting 70 μl of sterile PBS, followed by repeated aspiration and agitation 20 times with a pipette tip. A 1:10 dilution of the vaginal lavage fluid was made by transferring 50 μl of the fluid to SDA. Colony was counted after 72 h of incubation at 35 °C.

2.7. Gram staining

First, 10 μl of the lavage fluid was transferred onto a glass slide and gently spread using a pipette tip. The smear samples were then preserved with an alcohol burner and stained with a Rapid Gram Stain kit (Baso, BA-4012, China). Fungal morphology was observed and recorded at a 400 × magnification by a bright-field microscope (Olympus, BX53, Japan).

2.8. Histopathology

The vaginal tissues were fixed in 10% paraformaldehyde for more than 24 h and dehydrated in standard graded alcohol (70%, 85%, 95% and 100%) and then embedded in LEICA Paraffin (LEICA, 39601006, Germany). Vaginal tissues in paraffin were sectioned longitudinally to obtain sections with a 4 μm thickness for PAS staining (Baso, BA-4080B, China) and H&E staining (Beyotime, C0105, China). The slides were covered with neutral balsam (Guoyao, 10004160, China) and observed under a microscope (Olympus, BX53, Japan).

2.9. SEM

The specimens were fixed overnight in 2.5% glutaraldehyde, rinsed 3 times with PBS, fixed again in osmic acid, dehydrated in graded alcohol (30%, 50%, 70%, 80%, 90% and 100%), transitioned to isoamyl acetate and dried using the critical point drying method. The dried samples were glued onto SEM stubs, sputter-coated with a 10 nm thick layer of gold, and examined using a scanning electron microscopy (Hitachi, S-4800, Japan).

Table 1
Primer sequences used in the RT-PCR analysis.

Gene	Accession number	Sequence of primers	Size(bp)
Dectin − 1	NM_001309637.1	Forward: TTCTCAGCCTTGCCTTCCTA Reverse: TACGGTGAGACGATGTTTGG	179
Syk	NM_0011198977.1	Forward: GAGTCCTGGATGCTGGTGAT Reverse: TGTGTGACCAGAAGCACGTT	191
CARD9	NM_001037747.1	Forward: GGAGCTGCTGTGGGAACTAC Reverse: TCCGTAGGGAGAAGATGGTG	185
NF-κB	NM_009399.3	Forward: AAGACGGTGCTGGAGTCTGT Reverse: AGAGGTCTCCTTGCGTCTCA	114
β-actin	NM_007393.5	Forward: ATATCGCTGCGCTGGTCG Reverse: CGATGGAGGGGAATACAGCC	93

2.10. Luminex assay for serum markers

Serum samples were thawed at room temperature, and the concentrations of serum markers were measured by a Milliplex MAP Magnetic Bead Panel (Millipore, MTH17MAG-47K, USA) on the Luminex platform (Milliplex® Magpix, MAGPX15323707, USA) according to the manufacturer's protocol. The data were analyzed by Milliplex Analyst Version 5.1.

2.11. Real-time polymerase chain reaction (RT-PCR)

Total RNA from homogenized vaginal tissues was extracted with a Takara MiniBEST Universal RNA Extraction kit (Takara, 9767, Japan). Reverse transcription was carried out using 1.5 μg of total RNA with a FastKing RT kit(TIANGEN, Q5328, China) in a volume of 20 μl. RT-PCR was performed using a Talent qPCR PreMix kit (TIANGEN, FP209-02, China). A 1 μl volume of cDNA was amplified with specific premier of Dectin-1, Syk, CARD9 and NF-κB (Table 1) and normalized with β-actin. All the experiments described above were performed in triplicate. Relative gene expression was quantified by using the $2^{-\triangle\triangle Ct}$ method.

2.12. Western-blot

The vaginal tissues were homogenized in RIPA lysis buffer and PMSF liquor. The solution was then centrifuged twice at 12,000 rpm/min for 15 min at 4 °C. The supernatant was collected and the total protein concentration was quantified by a bicinchoninic acid (BCA) Protein Assay kit (Beyotime, P0012, China). The samples (20 μg) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes after electrophoresis. The membranes were blocked with 5% fetal bovine serum for 2 h at room temperature, followed by incubation with anti-Dectin-1 (1:2000, Abcam, ab140039, USA), anti-Syk (1:2000, Cell Signaling Technology, 13198, USA), anti-CARD9 (1:1000, Abcam, ab124922, USA), anti-NF-κB (1:2000, Cell Signaling Technology, 8242 S, USA) and anti-β-actin (1:1000, Cell Signaling Technology, 4970, USA) antibodies overnight at 4 °C. After incubation with a HRP-conjugated antibody (1:1000, Cell Signaling Technology, 7074, USA), the membranes were visualized with a chemiluminescence (ECL) kit (Bio-Rad, 170–5060, USA) via Image Lab Version 5.0. The expression levels were quantified by densitometry using Image J software.

2.13. Immunohistochemistry

The paraffin sections described in section 2.8 were deparaffinized, rehydrated and incubated in ethylenediamine tetraacetic acid (EDTA) (Beijing Zhongshan Jinqiao Biotechnology Inc., ZLI-9067, China) for 2.5 min in a pressure cooker. The slides were immersed in 3% H_2O_2 for 8 min to quench endogenous peroxidase activity. The tissues were then incubated with anti-Dectin-1 (1:300, Abcam, ab140039, USA), anti-Syk (1:300, Cell Signaling Technology, 13198, USA), anti-CARD9 (1:500, Abcam, ab124922, USA) and anti-NF-κB (1:800, Cell Signaling Technology, 8242 S, USA) antibodies overnight at 4 °C. The following day, the sections were incubated with secondary antibodies (Cell Signaling Technology, 8114, USA) for 20 min; substrate [3,3'-diaminobenzidine(DAB)] (Beijing Zhongshan Jinqiao Biotechnology Inc., ZLI-9017, Beijing) was added and the sections were counterstained with hematoxylin for 15 min at room temperature. The immunoreactions were mounted with neutral balsam and observed using a microscope (Olympus, BX53, Japan). The integrated optical densities (IOD) were semi-quantitatively analyzed with the aid of Image Pro-Plus 6.0 software.

2.14. Statistical analysis

All statisticals analyses were performed using SPSS 22.0 software. The data are expressed as the mean ± standard deviation (SD). One-way ANOVA was utilized for multiple group comparisons. When significant differences were indicated by ANOVA, inter-group comparisons were made using the least significant difference test (LSD-t). $P < 0.05$ was considered significant.

3. Results

3.1. PD treatment did not lead to weight loss during the treatment course

We first assessed the body weight of the animals throughout the treatment course. Body weight is a key indicator associated with drug safety. In the Model group, the body weight values were significantly lower than those in the PD group (20 g/kg) ($P = 0.02$ on Day 1) and the Fluconazole group ($P = 0.005$ on Day 4) (Fig. 2). There were no differences on –Day 3, -Day 1, Day 0, Day 7 and Day 14 in all groups. However, all groups showed a trend towards increasing body weight as time progressed and the Model group had significantly lower body weights than did the other groups throughout the experiment, although no statistical significance was detected.

3.2. PD decreased the colony counts of C. albicans

Next, we assessed the antifungal effects of PD in vaginal infection by quantifying the colony counts (Fig. 3). Compared with the Model group, all three PD groups and the Fluconazole group exhibited markedly reduced fungal loads from Day 7 onwards (Day 7, $P = 0.000$; Day 14, $P = 0.000$). PD treatment reduced the fungal burden in a dose-dependent manner until Day 14, while the vaginal colonization in the Model group persisted at the 10^4 to 10^5 level after inoculation.

3.3. PD inhibited the formation of hyphae in vaginal excretions

Gram staining is the most common initial stain used for the rapid identification of fungal hyphae (Vengayil et al., 2009). As shown in

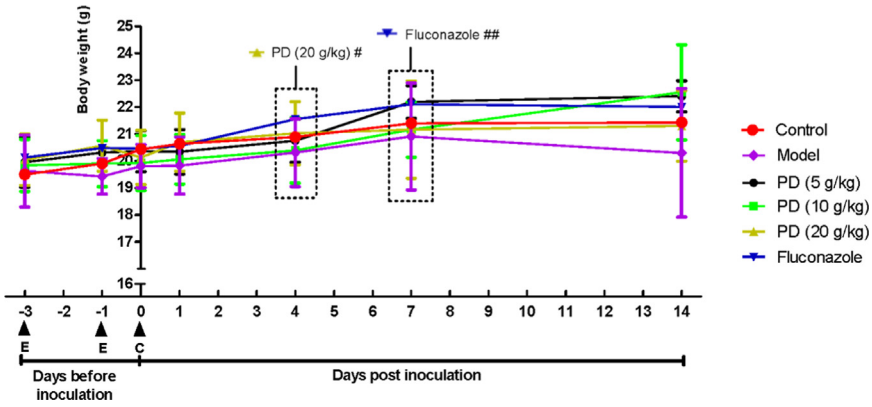

Fig. 2. Body weight throughout the treatment course. Estradiol benzoate was injected on -Day 3 and -Day 1. *C. albicans* was infected on Day 0. E: Estradiol benzoate, C: *C. albicans*. N = 15/group on –Day 3, -Day 1, Day 1 and Day 4, N = 10/group on Day 7, N = 5/group on Day 14. Each value is presented as the mean ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the Model group.

Fig. 4, in the Model group, a large number of hyphae was shown to intertwine with epithelial cells across all time points, while the number of hyphae in all three PD groups and the Fluconazole group gradually decreased over time. No hyphae were observed in the Control group.

3.4. PD reduced the adhesion of microorganisms to the vaginal tissue surface

PAS staining was performed to maximize the recovery and visualization of microorganisms adhered to the vaginal epithelium. The control group showed no visible microorganisms. However, the Model group exhibited severe infections marked by numerous pseudohyphae and blastoconidia during the whole treatment course. With PD treatment, the visible microorganisms gradually decreased at variable speeds over time (Fig. 5). SEM is a powerful tool for observing ultrastructure under a high-power microscope. Fig. 6 shows SEM images of vaginal tissues on Day 14. Abundant adherent yeasts and hyphae can be observed in the Model group, while a smooth and regular structure was observed in the Control group. A small number of hyphae were present in the PD group (5 g/kg). In the other two PD treatment groups and the Fluconazole group, varying degrees of scattered adhesions were also observed.

3.5. PD decreased serum inflammation levels

To assess the level of inflammation induced by infection, serum was collected on Day 14 and the levels of IL-10, IL-23 and TNF-α were analyzed by a Luminex assay (Fig. 7). The serum level of IL-23 was highest in the Model group (compared to the Control group) ($P < 0.01$) and lowest in the PD group (20 g/kg) and Fluconazole group (compared to the Model group) ($P < 0.01$). PD group (5 g/kg) and PD group (10 g/kg) also exhibited marginally decreased levels of IL-23 compared to the Model group. No significant differences were detected for IL-10 and TNF-α, although the PD-treatment groups and the Fluconazole group showed a trend towards reduced levels.

3.6. PD treatment reduced inflammation and maintained tissue integrity in vaginal tissues

To assess the tissue pathology, H&E staining was performed to evaluate inflammation in the vaginal tissue. Fig. 8 demonstrated that on Day 14, the vaginal tissues in the Model group were characterized by severe neutrophil infiltration and microabscesses in the mucosa. The degree of inflammation in the PD and Fluconazole treatment groups was mitigated, with reduced neutrophil invasion and majority of the tissue structure remained intact.

3.7. PD regulated the gene expression of components of the Dectin-1 signaling pathway

To identify the underlying molecular mechanism responsible for the antifungal and anti-inflammatory effects of PD, we investigated the expression levels of essential genes for Dectin-1 signaling pathway activation (Fig. 9). The model group exhibited significantly higher levels of Dectin-1, CARD9 and NF-κB expression than did the Control group ($P < 0.05$, $P < 0.05$, $P < 0.01$). After intervention with PD, all three genes were downregulated [Dectin-1: $P < 0.05$ for the PD group (5 g/kg) and PD group (20 g/kg); CARD9: $P < 0.01$ for the PD group (5 g/kg), $P < 0.05$ for the PD group (20 g/kg) and NF-κB: $P < 0.01$ for the PD group (10 g/kg), $P < 0.05$ for the PD group (20 g/kg)], while the expression level of Syk remained unchanged regardless of treatment.

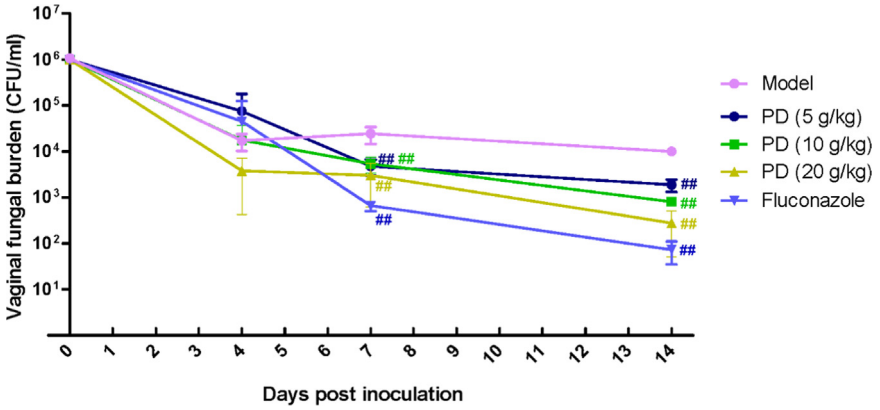

Fig. 3. Vaginal fungal burden changes during the treatment course. N = 15/group on Day 4, N = 10/group on Day 7, N = 5/group on Day 14. Each value is presented as the mean ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the Model group.

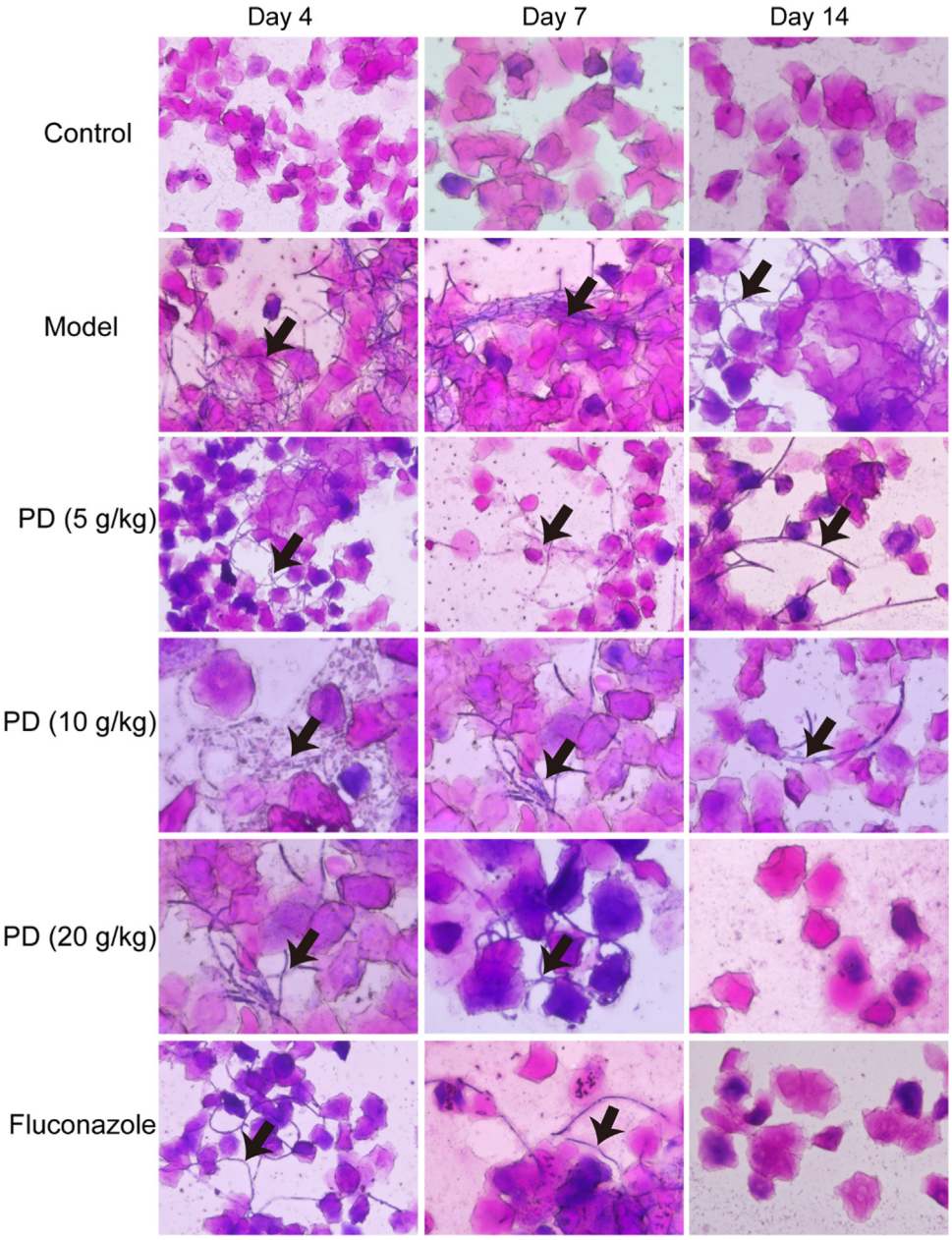

Fig. 4. Decreased formation of hyphae in vaginal excretions after PD treatment, as indicated by Gram staining (400 ×). The arrows represent visible hyphae in vaginal excretions.

3.8. PD regulated the protein expression of components of the Dectin-1 signaling pathway

We also confirmed this regulation at the protein level by Western Blot. As shown in Fig. 10, the expression levels of Syk, CARD9 and NF-κB were significantly upregulated in the Model group, while the PD and Fluconazole treatments downregulated the expression of these proteins at variable levels, especially for NF-κB ($P < 0.01$). Dectin-1 expression was increased in the Model group compared with that in the Control group but reduced in the PD group (5 g/kg) and PD group (20 g/kg), although the differences were not significant.

3.9. Immunohistochemical analysis

Immunohistochemical assays were performed to evaluate the protein levels of Dectin-1, Syk, CARD9 and NF-κB on Day 14 (Fig. 11). The immunohistochemistry data showed that Dectin-1, CARD9 and NF-κB were significantly elevated in the mucosa and submucosa, while the Syk protein was mainly expressed in the submucosal layer. The upregulation was highest in the Model group, as shown by the staining. Treatment with PD or fluconazole significantly reduced the levels to varying degrees with statistical significance.

4. Discussion

VVC causes discomfort in patients, whose quality of life is severely compromised. The high occurrence and recurrence rates of VVC also pose threats to public health and have been a focus of recent research. This study focused on utilizing PD from TCM as a novel therapeutic agent for treating VVC and we found that the administration of PD not only decreased fungal loads and adhesion but also eased inflammation and damage in the vaginal tissue. In addition, the levels of inflammatory factors, including IL-10, IL-23 and TNF-α, were inversely correlated with PD treatment, although no statistical significance was

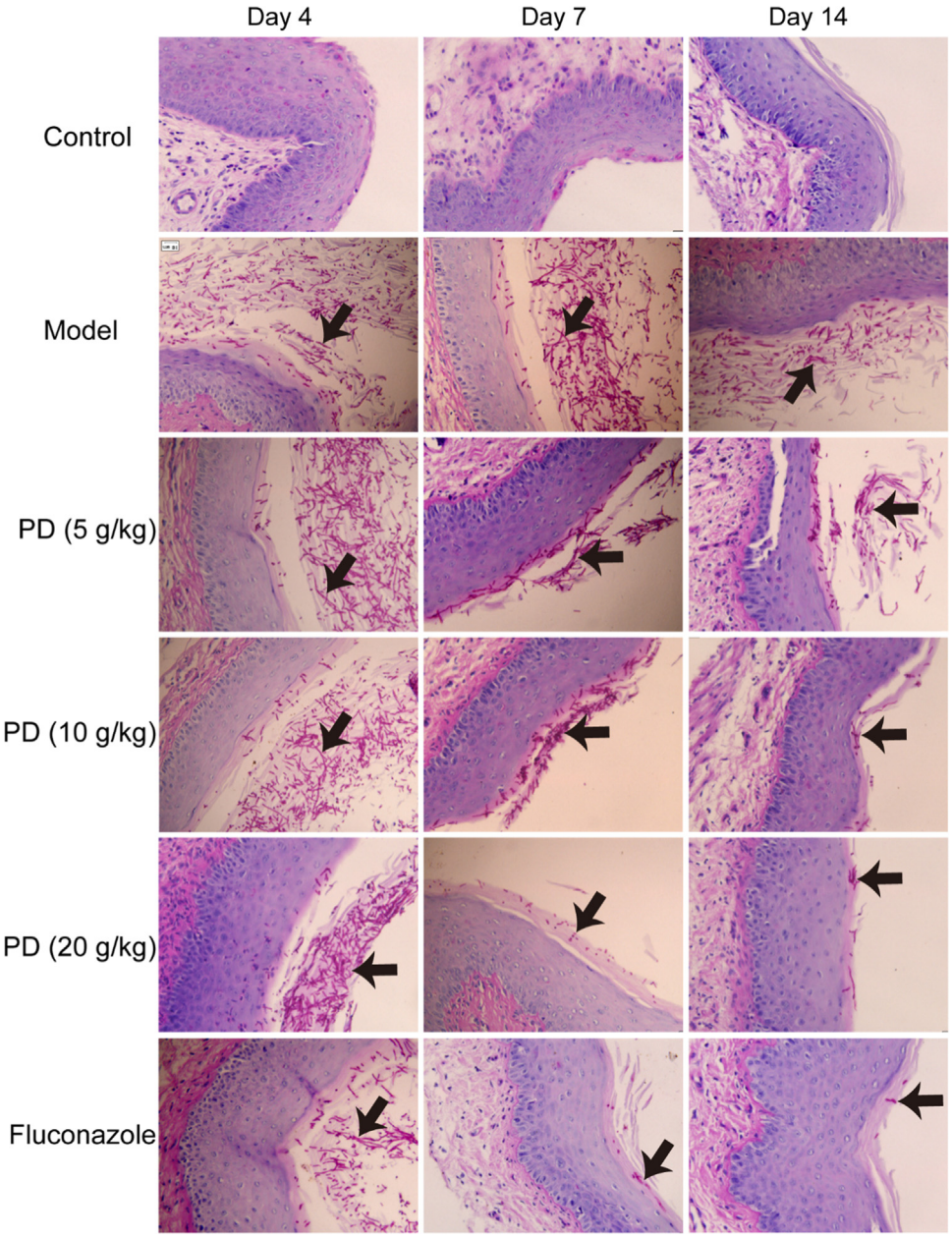

Fig. 5. Reduced fungal adhesion to the vaginal epithelium after PD treatment, as indicated by PAS staining (400 ×). The arrows represent visible pseudohyphae and blastoconidia in the vaginal epithelium.

observed for IL-10 and TNF-α. Further investigation of the mechanism revealed that the anti-*C. albicans* effects of PD was closely associated with inhibition of the Dectin-1 signaling pathway at both the protein and gene levels. The finding obtained in this study help to establish an experimental basis for the use of PD as a promising treatment for VVC.

To explore the therapeutic effects of PD in depth, we employed the estrogen-dependent vaginal candidiasis mouse model, which is considered a highly reliable model for *in vivo* testing of potential anti-*C. albicans* therapies. The high dose of estrogen required in this model promotes epithelial thickening by increasing epithelial proliferation and accelerating *C. albicans* adherence to the vaginal wall and subsequent colonization. Moreover, elevated glycogen levels caused by enhanced estrogen levels in the tissues also allows *C. albicans* to proliferate by providing additional nutrients (Buchanan et al., 1998; Cunha et al., 2004; Redondo-Lopez et al., 1990). Under estrogen-induced conditions, animals inoculated with *C. albicans* can carry a detectable vaginal fungal burden for weeks or even months (Jr and Sobel, 1999; Yano and Jr, 2011). Consistent with previous studies (Li et al., 2012; Yano and Jr, 2011), the colony counts in this study were maintained at 10^4 to 10^5 throughout the 14-day treatment course and validated the VVC model under physiological conditions.

While PD has been prescribed in TCM for centuries, the exact pharmacological and toxicological profiles remained unclear. It is essential to establish a safety profile for PD in an animal model to balance the adverse effects and therapeutic effects. Body weight is a widely used parameter to assess adverse effects in animal models. Animals experiencing severe adverse effects often exhibit body weight loss. Consistent with our findings, a previous report (Mosci et al., 2013) also revealed no evident changes in body weight in the VVC mouse model throughout the experiment. In our study, the body weight value of the Model group was slightly lower than those of the other groups throughout the experiment, with no statistically significant differences; this effect may have been caused by the inflammation and stress induced by infection and estrogen. Although more detailed toxicological studies in the future

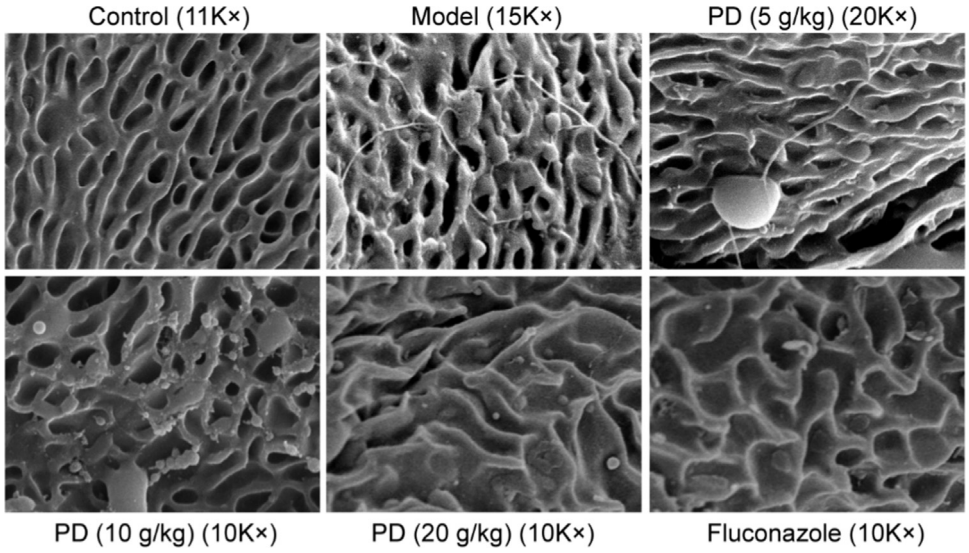

Fig. 6. Morphology of the vaginal epithelium by SEM after infection and PD treatment. 20 K× : 20,000 × magnification; 15 K× : 15,000 × magnification; 11 K× : 11,000 × magnification; 10 K× : 10,000 × magnification.

will allow a more comprehensive analysis to identify any potential adverse effects, the weights of all PD treatment groups were general steady across the time points, suggesting that PD does not induce significant adverse effects, even at high doses and is safe to apply in animals to ameliorate infection.

One essential measurement for determining anti-microbial effects is the inhibitory effects on the proliferation and adhesion of the microorganism. As demonstrated by colony counts, PD treatment significantly reduced the *C. albicans* load from Day 7 onwards, indicating that a prolonged treatment may be beneficial for fungal clearance. Gram staining images confirmed that fungal survival was reduced by PD. The results of PAS staining and SEM also revealed reduced adhesion of *C. albicans* in the PD treatment group based on the vertical dimension and the morphological appearance of the vaginal tissue, respectively. The above findings supported the conclusion that PD effectively decreases fungal proliferation and adhesion *in vivo*.

Like other infectious diseases, VVC elicits a protective immune response in the host, accompanied by tissue inflammation. Elevated levels of inflammatory cytokines in the circulation serve as indicator of an immune response. Accumulated evidence shows that Th17 cells play a predominant role in coordinating protective mucosal immune responses against candidiasis (Conti et al., 2009; Eyerich et al., 2008). By inducing neutrophil-activating factors, inflammatory chemokines and antimicrobial proteins that ameliorate infection, the Th17 response is a powerful defense against fungal infection. Another indicator of an activated immune response is TNF-α. The level of TNF-α is often elevated concurrently with IL-23 during a robust Th17 response (Filler, 2012) (Fsm et al., 2017). On the other hand, anti-inflammatory cytokines, such as IL-10, tune down immune responses to pathogens to prevent extensive tissue damage in the host (Saraiva and O'Garra, 2010). IL-10 shows strong inhibitory effects on defense against *C. albicans* infection (Netea et al., 2004). A study performed by De et al. (De et al., 2013) in the VVC mouse model revealed that IL-10-deficient mice suffered from severe tissue inflammation and had extensive leukocyte infiltration, even though fungal growth was limited during primary infection and re-challenge. In this study, serum levels of IL-23 were significantly lower in the PD groups than those in the Model group; levels of IL-10 and TNF-α were also lower in the PD groups than those in the Model group, although this difference was not significant. Additionally, we further assessed inflammation by examining histological profiles after H&E staining, and reduced damage was observed in mice treated with PD. This evidence indicated that PD alleviates infection and inflammation and thereby prevents damage to the host.

The combined results from colony counts, Gram staining, PSA staining, SEM, serum makers and H&E staining indicate that PD exerts its curative effects on *C. albicans* infection via multiple angles. First, PD may prevent pathogen proliferation. Next, PD may interrupt the adhesion process, which is essential for persistent infection, and alleviate inflammation and tissue damage. Therefore, PD may act as an effective therapy for the clearance of fungal infections. Previous research (Xia

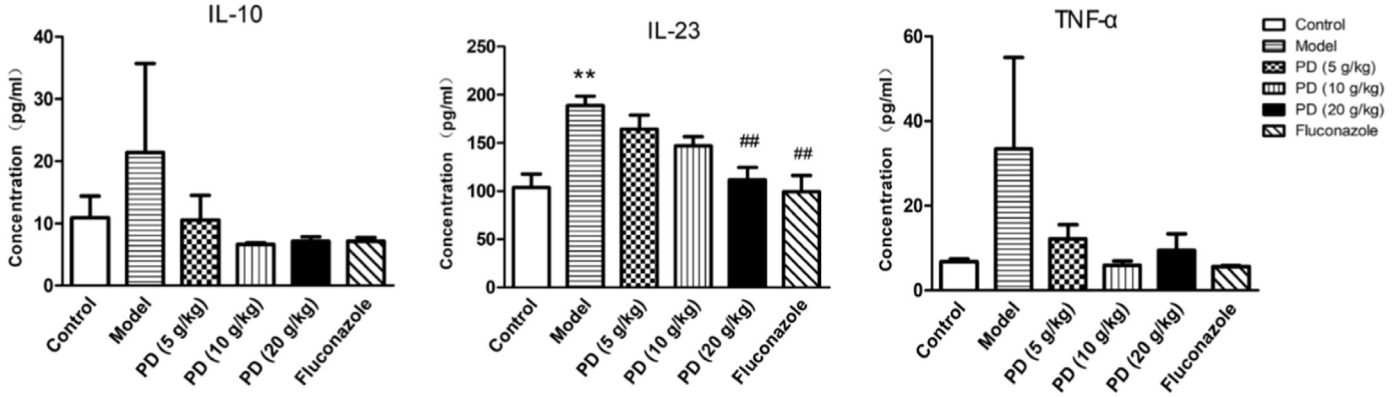

Fig. 7. Reduction of inflammatory cytokines in the serum after PD treatment. N = 5/group. Each value is presented as the mean ± SD. *P < 0.05, **P < 0.01 versus the Control group; #P < 0.05, ##P < 0.01 versus the Model group.

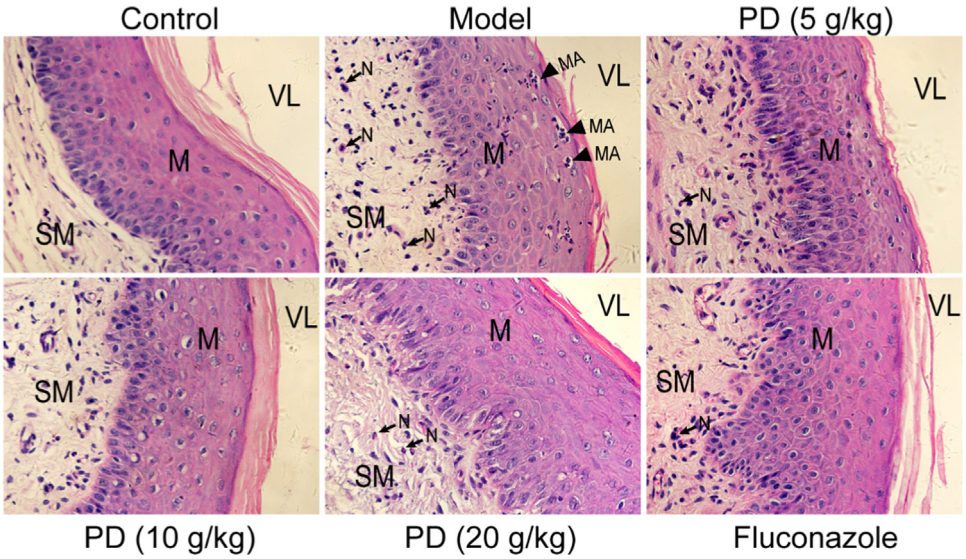

Fig. 8. PD improved tissue integrity and reduced neutrophil infiltration in vaginal tissues (400 ×). The arrows represent neutrophils (N). The triangles represent microabscesses (MA). VL: vaginal lumen; M: mucosa; SM: submucosa.

et al., 2016) reported that PD could inhibit the *C. albicans* proliferation and reduce inflammatory cytokines in VVC mice; however, the intervention time of PD was 7 Days with one observation point. In addition, the weight of the mice during the treatment course and the adhesion of *C. albicans* to the vaginal epithelium were not investigated. In this paper, we carried out a various-aspect study on the effects of PD on VVC based on mouse body weight, proliferation, adhesion and inflammatory factors at three time points over 14 days.

In many studies of TCM regimens, an unidentified mechanism is the main barrier that prevents wide application. Based on the findings of reduced pathogen proliferation, adhesion and inflammation after PD treatment, we hypothesized that the therapeutic effects of PD may be related to central pathways that regulate host-pathogen interactions and recognition. The first step of *Candida* infection in the vagina

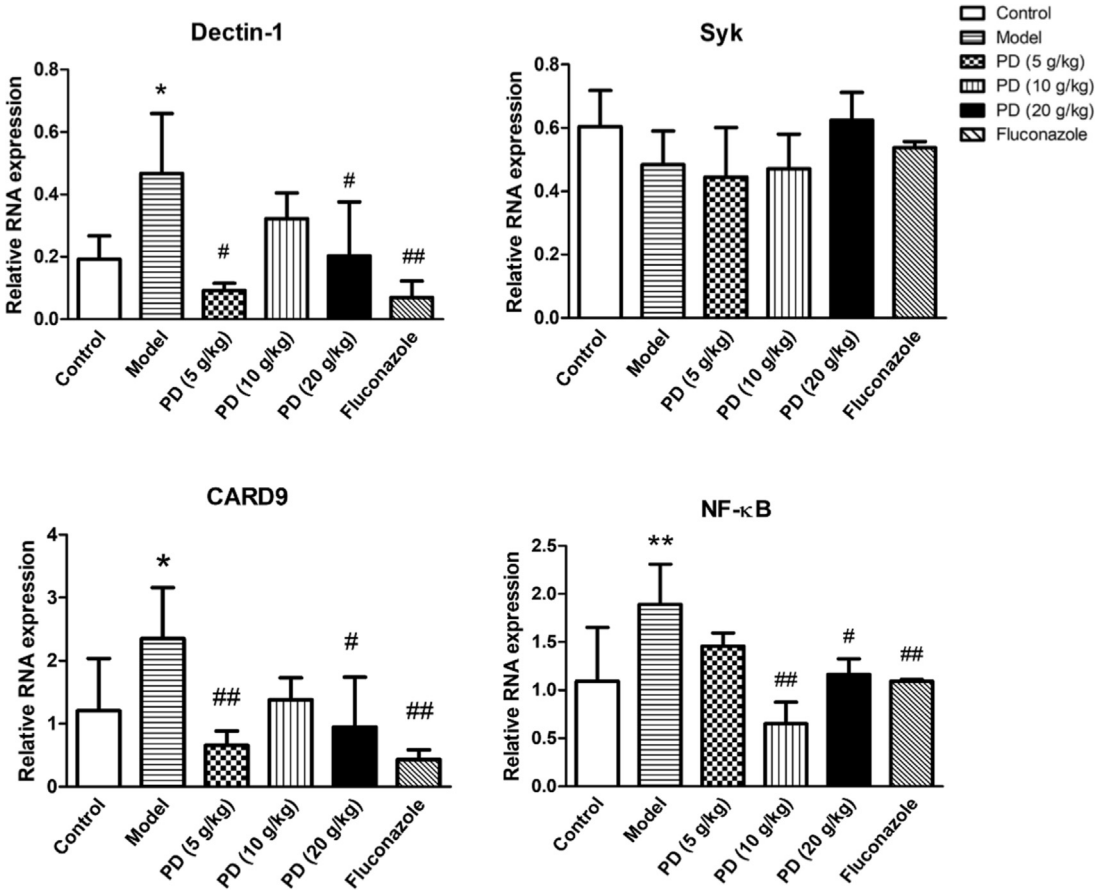

Fig. 9. PD treatment down-regulated the gene expression of Dectin-1, CARD9 and NF-κB. N = 5/group. Each value is presented as the mean ± SD. *P < 0.05, **P < 0.01 versus the Control group; #P < 0.05, ##P < 0.01 versus the Model group.

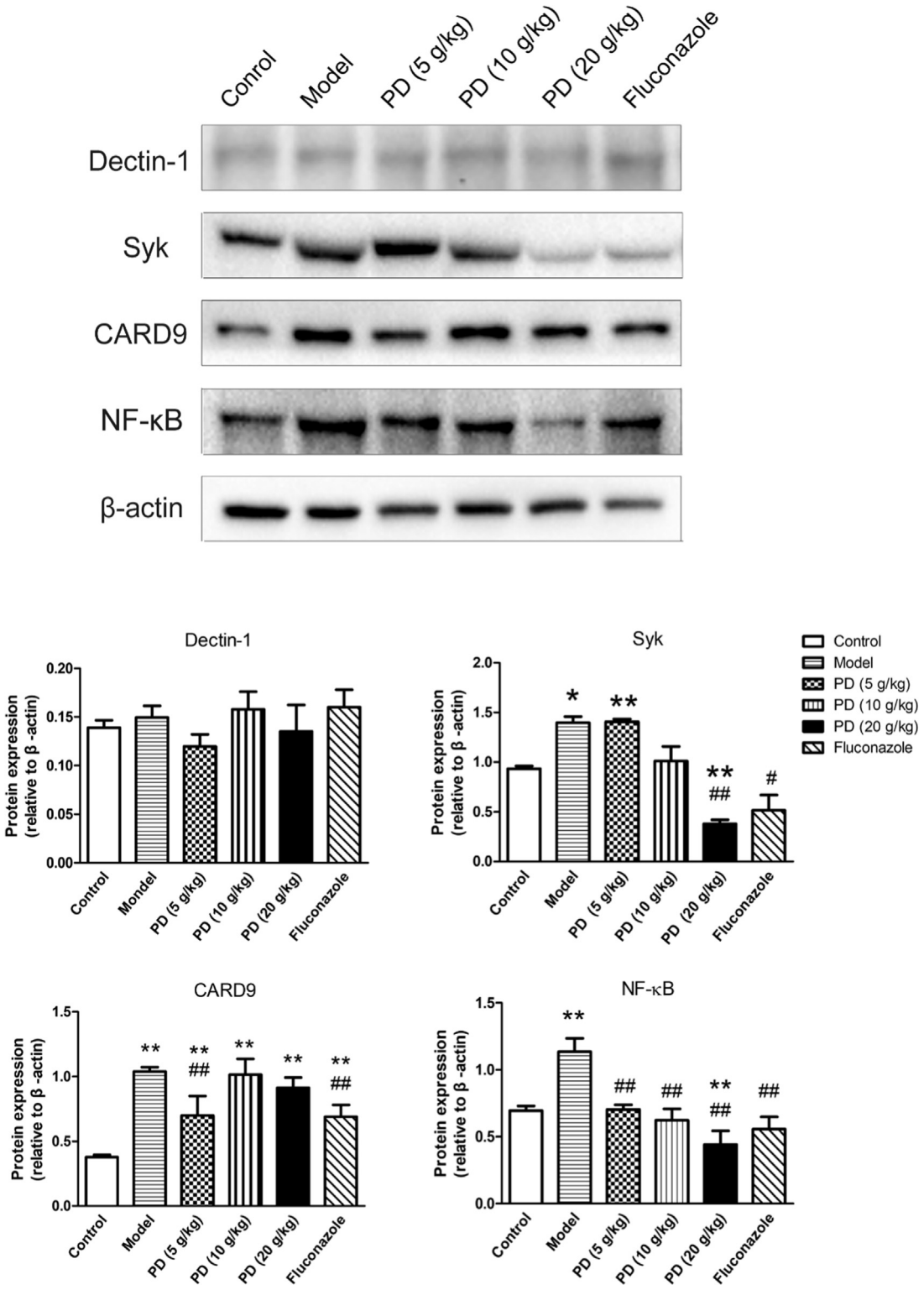

Fig. 10. Effect of PD on the protein expression of Dectin-1, Syk, CARD9 and NF-κB. Protein expression levels were analyzed by Western Blot. N = 5/group. Each value is presented as the mean ± SD. *P < 0.05, **P < 0.01 versus the Control group; #P < 0.05, ##P < 0.01 versus the Model group.

involves adhesion of the pathogen to the epithelial layer. The fungal cell wall consists mainly of carbohydrates, including mannose-based structures, β-glucan, chitin and other components, of which β-glucan comprises up to 50% of the dry weight of the fungal wall and is an essential structural component that provides elasticity and mechanical strength (Calderone and Braun, 1991). These unique structures are recognized by the host immune system as foreign antigens via PAMP-PRR interactions. Hence, β-glucan plays an essential role in antifungal immunity (Taylor et al., 2007). Dectin-1 is the major PRR that recognizes β-glucan in mammalian cells (Ariizumi et al., 2000; Brown and Gordon, 2001). Taylor et al. showed that Dectin-1$^{-/-}$mice are susceptible to infection with *C. albicans*, which is related to impaired cytokine production and poor neutrophil-mediated fungal eradication (Taylor et al., 2007). Another study in humans obtained similar results, as women

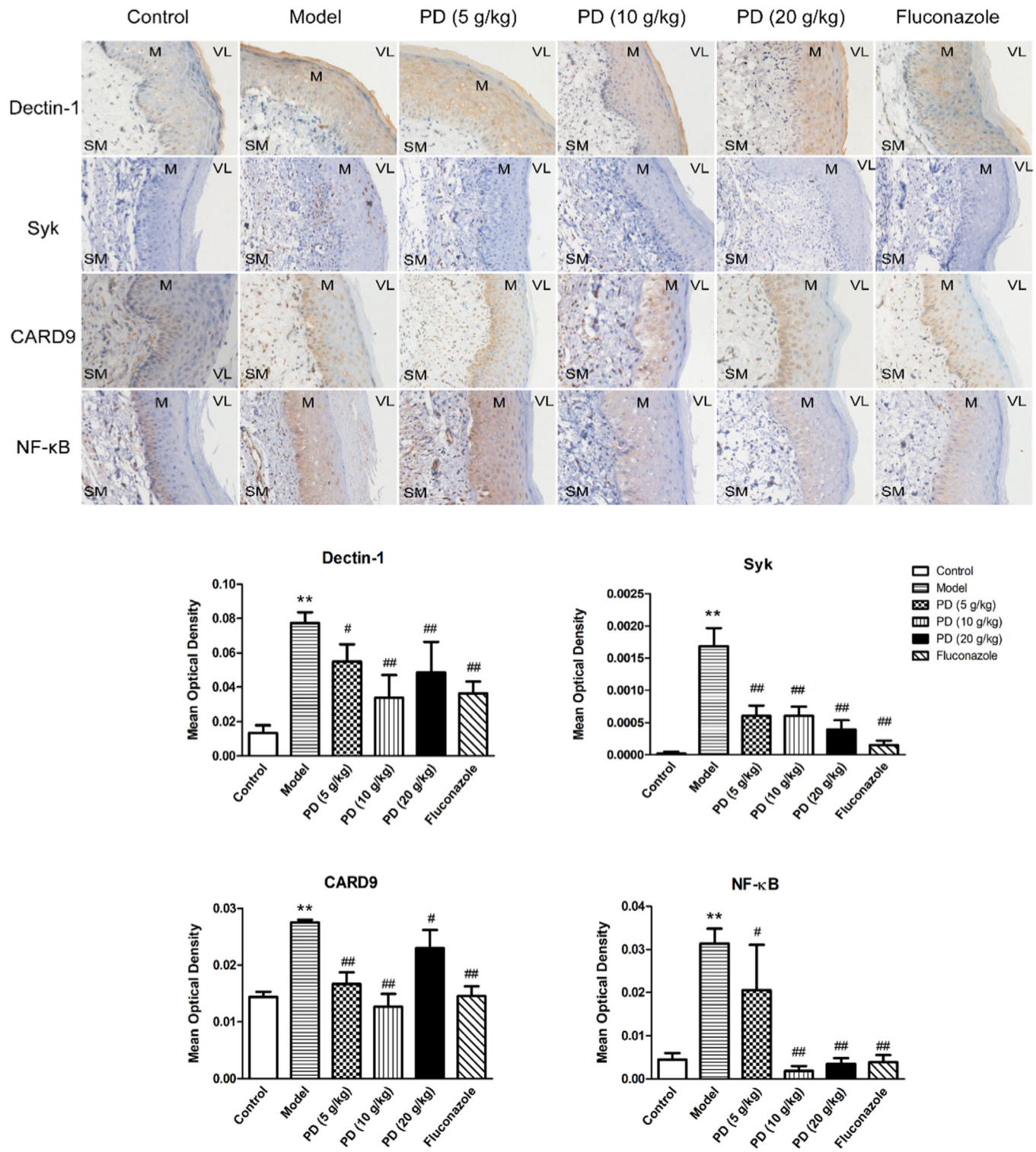

Fig. 11. Immunohistochemistry was performed to evaluate the protein expression levels of Dectin-1, Syk, CARD9 and NF-κB in vaginal tissues (200 ×). VL: vaginal lumen; M: mucosa; SM: submucosa. N = 5/group. Each value is presented as the mean ± SD. *P < 0.05, **P < 0.01 versus the Control group; #P < 0.05, ##P < 0.01 versus the Model group.

with a gene deficit in Dectin-1 exhibited impaired cytokine production and increased susceptibility to familial chronic mucocutaneous candidiasis and recurrent VVC (RVVC) (Ferwerda et al., 2009). When activated by ligand binding, Dectin-1 can recruit Syk via a single phosphotyrosine, which in turns activates the transcription factor NF-κB through CARD9 (Drummond and Brown, 2011). Researchers (Glocker et al., 2009; Gross et al., 2006) found that CARD9$^{-/-}$ mice have markedly increased susceptibility to disseminated candidiasis. To investigate whether the Dectin-1 signaling pathway is involved in persistent infection of *C. albicans*, the mRNA and protein expression levels of Dectin-1, Syk, CARD9, and NF-κB were measured by RT- PCR, Western Blot and immunohistochemistry. Our results showed that these targets were upregulated in Model group compared with their expression in the Control group, suggesting that the Dectin-1-Syk-CARD9-NF-

κB signaling pathway was activated in the VVC mouse model. After PD administrating for 2 weeks, the levels of both mRNA and protein were decreased to different degrees, implying that PD effectively reduced fungal infection and tuned down the Dectin-1 signaling pathway. However, we observed inconsistencies between Syk mRNA and protein levels. This finding may be attributed to either potential microRNA-based regulation or ubiquitin-based degradation (de Sousa et al., 2009). Moreover, Syk is regulated by multiple signaling pathways during the process of fungal detection (Kingeter and Lin, 2012), and it remains unknown whether crosstalk between these pathways contributes to these inconsistencies.

The findings of the current study have several limitations. Firstly, PD is a classical prescription in TCM with different chemical compositions and the absorbed bioactive components from PD that attenuate VVC remain unclear. The effective components of PD need to be elucidated. In addition, the downstream targets of the Dectin-1 signaling pathway are also regulated by other elements (Kingeter and Lin, 2012). A thorough study of the downstream mechanism is essential for future investigations.

In summary, this study illustrated the therapeutic efficiency of PD for VVC *in vivo* and the potential mechanism might involve suppressing the activation of the Dectin-1 signaling pathway. This study establishes an experimental basis for PD as a potentially promising therapeutic option for the treatment of VVC.

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Author contributions

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Analyzed the data: Haiwang Wu, Qingying Yu, Jie Gao

Wrote the manuscript: Lilin Yang, Jie Gao, Songing Luo

Conflicts of interest

The authors declare that there are no conflicts of interest.

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