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Toxicity Evaluation of Honghe Fujie lotion Against *Candida Albicans* in Vitro and in Vivo

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Abstract: Mycotic vaginitis is a common vulvovaginitis in gynecology, with a high incidence rate, which is mainly caused by *candida albicans* infection. Honghe Fujie lotion is mainly used to treat fungal vaginitis caused by *C. albicans*, but its antibacterial mechanism against *C. albicans* in vitro and in vivo is still unclear. Here, we found that Honghe Fujie lotion can significantly inhibit the formation of *C. albicans* ATCC (American Type Culture Collection) 10231 colony, and its inhibitory effect on colony formation continued to increase with increasing drug concentration. The group treated with Honghe Fujie lotion showed a dose-dependent decrease in vaginal mucosal damage in VVC (vulvovaginal candidiasis) model rats, hindered the infiltration and diffusion of inflammatory cells, and exhibited similar conditions to the positive control drug Amphotericin B group. In summary, our data indicated that Honghe Fujie lotion can significantly reduce the formation of *C. albicans* in the vagina of VVC model rats, protected the vaginal mucosal tissue of rats, hindered the infiltration and diffusion of inflammatory cells, and improved the vaginal infection status of VVC model rats.

Keywords: Honghe Fujie Lotion; *Candida Albicans*; Mycotic Vaginitis.

1. Introduction

Mycotic vaginitis is a common and frequently occurring disease that endangers the health of women. In recent years, the incidence rate of mycotic vaginitis and non-specific vaginitis was on the rise [1]. At present, imidazole broad-spectrum antibiotics and antifungal chemicals are mainly used in the market to treat such diseases, including clotrimazole vaginal tablets, econazole nitrate suppository, and miconazole nitrate gel. These drugs have certain antibacterial and anti-inflammatory effects, which can alleviate patients' discomfort in a short time, but long-term use will cause abnormal proliferation of normal flora in the human body [2].

C. albicans has been identified as a common pathogen in humans and can settle in different locations of healthy hosts, especially in the gastrointestinal, skin, and reproductive tracts [3]. Infection caused by *C. albicans* may come from the activation of virulence characteristics of normal *C. albicans* strains and interference with host defense systems [4]. The toxicity of *C. albicans* is reflected through its adhesion to host cells, biofilm formation, and secretion of hydrolytic enzymes [5], and it can cause skin surface infections and systemic infections. VVC is a type of vulvovaginal inflammation mainly caused by *C. albicans* infection.

Research has confirmed that Honghe Fujie lotion can exert insecticidal, antipruritic, detoxifying and dehumidifying effects by inhibiting the development and growth of *C. albicans* [6], and has antibacterial, anti-inflammatory and antipruritic effects [7]. According to the latest clinical trials, Honghe Fujie lotion can inhibit the growth and reproduction of *C. albicans* to a certain extent in the body, and can also act to inhibit bacterial growth and reproduction in vitro [8]. At present, research has determined the activity of Honghe Fujie lotion in vitro through micro dilution method, indicating that it has strong anti *C. albicans* activity [9], but its antibacterial mechanism is still unclear and further research is needed. As of now, the material basis for the anti-inflammatory and antibacterial effects of Honghe Fujie lotion was still unclear. This drug has been used in clinical practice for many years, but the evaluation of its in vivo and in vitro toxicity was still very limited.

This study focuses on the in vivo and in vitro antibacterial effects of Honghe Fujie lotion. The agar diffusion method was used to study the in vitro anti *C.* albicans effect of Honghe Fujie lotion. Subcutaneous injection of estradiol glutarate was given to rats, followed by injection of an appropriate amount of *C. albicans* suspension into the vagina of rats. The anti *C. albicans* effect of Honghe Fujie lotion in vivo was studied by establishing a model of mycotic vaginitis. This experiment further improved the basic research on antibacterial and anti-inflammatory effects of traditional Chinese patent medicines Honghe Fujie lotion, and it provided a research basis for the clinical medication safety evaluation of Honghe Fujie lotion, aiming to provide scientific basis for the in-depth development and utilization of this preparation and clinical safe medication.

2. Materials and Methods

2.1. Experimental Strains, Experimental Animals and Culture Media

C. albicans (strain number: ATCC 10231, batch number: A1036B), Clinical isolation of strains from the Experimental Center of the Medical Department of Xi'an Jiaotong University.

SPF grade SD strain female sterile rats aged approximately 7 weeks. The room temperature in the laboratory is about 30 ℃, with continuous 12 hours of light (8 am-8 pm) and a temperature and humidity of 50 ± 10%. After weighing, rats were divided into cages according to their body weight difference, and they were raised in a stable environment for 5

days and randomly divided into groups according to their body weight.

Preparing agar medium and Amphotericin B with a final concentration of 2 μg · mL^{-1}.

2.2. In Vitro Culture of C. albicans Colony

After culturing at room temperature, a single colony was isolated and inoculated onto YPD (Yeast Peptone Dextrose) medium. The strain was incubated in a constant temperature water bath shaker at 90 rpm, and centrifuged at 4 ℃ and 3000 rpm to obtain the strain. Diluting the bacterial solution to 106 CFU · mL^{-1} using RPMI (Roswell Park Memorial Institute)-1640 liquid culture medium to form a standard suspension for future use.

Diluting the Honghe Fujie lotion with YPD culture medium to the original concentration of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128. Marking each row with a number and sequence, and used a micro sampler to add 100 uL of YPD in the middle of the tenth hole in each row. Adding 100 uL of colony suspension in sequence and perform a total of five biological replicates.

The final inoculation amount of the Honghe Fujie lotion was about 5 × 10^5 CFU · mL^{-1}, or 5 per hole × 10^4 bacteria. Shaking the microplate with a micro oscillator to mix the solution in each well. Placing a lid on the microplate and sealed it with adhesive paper. Incubating at room temperature for 20 hours.

Using a black light source as the background, observed the growth characteristics of the detection bacteria and standard bacteria in the growth control well, presented a PYD shape inside the well. At the bottom of the hole, obvious precipitation can be observed, with a significant decrease in turbidity as the endpoint.

2.3. Formation of Biofilm of C. albicans

Selecting 100 uL of bacterial colony suspension and inoculated it in a 96 well microculture plate. Incubating at room temperature for one day and one night, allowed *C. albicans* fully adhere to the substrate surface of the 96 well plate, and formed a biofilm structure of *C. albicans* can be identified using an inverted microscope the *C. albicans* biofilm.

Adding YPD culture medium and various dilutions of Honghe Fujie lotion to the established biofilm, and set 5 biological replicates for each group. 2 μg · mL^{-1} Amphotericin B was used as the positive control group, while the group without Honghe Fujie lotion was used as the negative control group.

Extracting the culture medium at the 8th, 24th and 48th hours of bacterial culture, and rinsed twice with sterile PBS. Injecting 100 uL of culture medium and 100 uL of XTT working solution into each well. Incubating at room temperature for 3 hours, and detected the absorbance value using a multifunctional enzyme-linked immunosorbent assay at 450nm, while determining the minimum adhesive inhibitory concentration.

2.4. Germination of C. albicans Germ Tubes

Taking 6 sterile labeled test tubes and added 500 μL YPD liquid culture medium, added an appropriate amount of Honghe Fujie lotion in sequence to dilute to a concentration gradient of 2 to 128 times that of the original solution. Positive control group added to 110 μL Amphotericin B and 500 μL YPD liquid culture medium. Adding 20 μL ATCC 10231 bacterial suspension, adjusted the volume to 1 mL with physiological saline. Placing the test tube in a culture incubator at 37 ℃ for 3 hours, observed and recorded the number of normal and inhibited germ tubes using a blood cell counting plate according to the white blood cell counting method. If the length of the germ tube was greater than or equal to twice the length of the bacterial body, it was considered a normal germ tube, and if it was less than twice the length, it was considered a suppressed germ tube. Inhibition rate of sprout tube germination= (number of inhibited sprout tubes in the experimental group/total number of sprout tubes in the group) × 100%.

2.5. Observation of the Morphology of C.albicans under Transmission Electron Microscopy after Administration

Adding ATCC 10231 bacterial suspension and Honghe Fujie lotion to the sterilization test tube, and adjusted the concentration to 2MIC (Minimum inhibition concentration). Incubation in a constant temperature water bath shaker at room temperature of 37 ℃, centrifuged for 10 minutes, then collected the bacterial body. Placing the bacterial body in 2.5% glutaraldehyde at room temperature for 2 hours, then transfer it to a 4 ℃ refrigerator for storage. Purifing and dehydrated the sample with ethanol, embed it, sliced it into ultra-thin sections, stained it, and observed the morphological and structural characteristics of the ATCC 10231 bacterial body under electron microscopy.

2.6. Modeling and Grouping

75 clean grade SD strain female rats (6-8 weeks old) were selected, and the other 45 rats were subcutaneously injected with estradiol glutarate every other day starting from the week before inoculation, keeped the mice in a state of pseudo estrus until the end of the experiment. After one week, the purified *C. albicans* strain was inoculated in culture medium and incubated in a water bath at 25 ℃ for 16-18 hours. On the 7th day, a 20ul bacterial suspension with a density of 2.5×10^6·L^{-1} (*C. albicans* spore inoculation amount was 5 × 10^4) dissolved in PBS buffer was injected into the vagina of rats, and the rats were inverted for 5 minutes. After successful modeling, rats exhibited redness and swelled of the external genitalia after 3-5 days, with a significant increase in vaginal secretions. In severe cases, curdled secretions could be discharged from the vaginal opening. After microscopic examination of the smear, pseudohyphae and budding spores could be seen. Randomly selected and divided the successfully modeled rats into three groups: model control group, Amphotericin B group, and Honghe Fujie lotion group (the high dose group was 1/5 of the original solution concentration, the medium dose group was 1/10 of the original solution concentration, and the low dose group was 1/20 of the original solution concentration). Administing 100 μL of Honghe Fujie lotion into the vagina of the Honghe Fujie lotion group rats for a week, injected 1.8 mg · kg^{-1} of Amphotericin B solution into the vagina of rats in the Amphotericin B group for a continuous week, injected 100 μL of physiological saline into the vagina of blank control group and model control group rats for one week.

2.7. Sampling and Microscopic Examination

On days 0, 4 and 8 after treatment, samples were taken. Five rats were randomly selected from each group, and the vagina was rinsed with 100 uL of PBS. The rinsed liquid was placed in a clean and sterile test tube, and 10 μL of the liquid

was diluted in 190 μL sterile saline. Inoculating on the culture medium, cultured at room temperature for one day and one night, and finally observed for colony formation.

2.8. Pathological Examination of Vaginal Tissue

Removing the vaginal tissue of the mouse and fix it in a 10% concentration of formalin solution. Examining its pathological tissue, embed the mouse in paraffin, sliced and stained with HE to observe the inflammatory tissue cells in the vaginal specimen of the rats.

2.9. Statistical Analyses

Statistical analyses of all data were conducted in Excel 365, using the Student's *t*-test to compare differences between the control and samples at 5% significance level.

3. Results

3.1. Inhibition of C. albicans Colony Formation in Vitro by Honghe Fujie lotion

Finally, it was determined that the MIC of Honghe Fujie lotion against ATCC 10231 suspended bacteria was 1/64 of the dilution of the original solution. The results showed that the Honghe Fujie lotion could significantly inhibit the formation of ATCC 10231 colony, and its inhibitory effect on colony formation continued to increase with the increasing concentration of the drug (Figure 1).

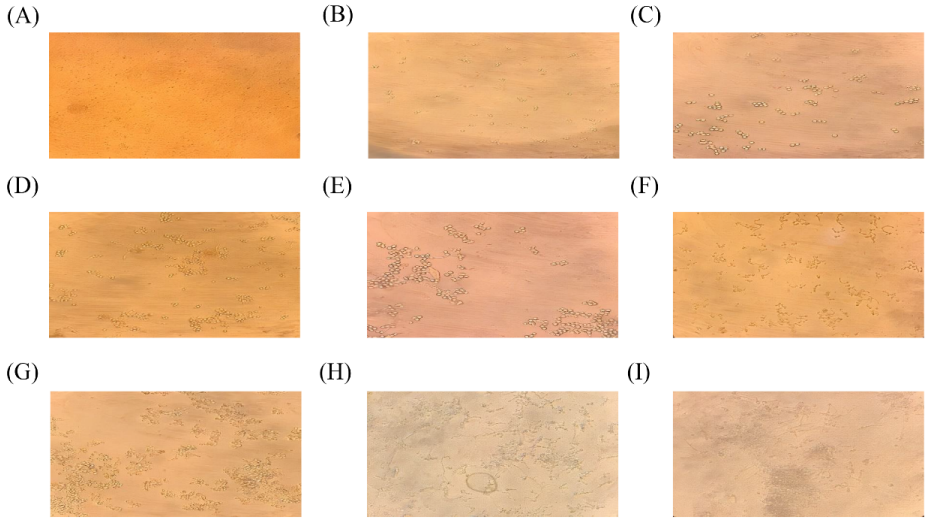


Figure 1. Inhibition of the growth of C. albicans in vitro by Honghe FuJie lotion (×100). (A) Fungal control group. (B) Original sample group. (C) The group diluted twice with Honghe Fujie lotion. (D-I) Diluting the groups 4 times, 8 times, 16 times, 32 times, 64 times and 128 times with Honghe Fujie lotion.

3.2. Inhibition of Different Stages of C. albicans Biofilm Formation by Honghe FuJie lotion

Table 1. Inhibition of C. albicans biofilm formation at different stages by Honghe Fujie lotion

Dilution ratio	The different periods of action of Honghe Fujie lotion								
	8 h			24 h			48 h		
	A_{450} Value	t Value	P Value	A_{450} Value	t Value	P Value	A_{450} Value	t Value	P Value
Positive control	0.3658 ± 0.0132	-	-	0.3923 ± 0.0023	-	-	0.4197 ± 0.0317	-	-
Negative control	0.9569 ± 0.0039	-	-	1.0289 ± 0.0211	-	-	1.1021 ± 0.0055	-	-
Stock solution	0.0958 ± 0.0013	177.9430	<0.001	0.1131 ± 0.0099	41.1820	<0.001	0.1256 ± 0.0094	86.964	<0.01
1/2	0.1198 ± 0.0075	125.4600	<0.001	0.1485 ± 0.0161	31.9940	<0.001	0.1670 ± 0.0289	21.527	<0.01
1/4	0.1520 ± 0.0051	167.5470	<0.01	0.1703 ± 0.0066	30.6240	<0.01	0.1858 ± 0.0414	10.972	<0.01
1/8	0.238 ± 0.0027	172.3250	<0.01	0.2821 ± 0.0056	26.8270	<0.01	0.2914 ± 0.0084	42.476	<0.01
1/16	0.3732 ± 0.0056	259.2600	<0.01	0.3986 ± 0.0089	17.5680	<0.05	0.4298 ± 0.0045	38.601	<0.05
1/32	0.4156 ± 0.0126	52.6310	<0.05	0.4801 ± 0.0036	15.0670	<0.05	0.4959 ± 0.0488	5.433	<0.05
1/64	0.4764 ± 0.0025	100.2370	<0.05	0.5021 ± 0.0283	4.7890	<0.05	0.5501 ± 0.0136	15.858	<0.05
1/128	0.7539 ± 0.0341	7.5340	<0.05	0.8013 ± 0.0169	5.2030	<0.05	0.8429 ± 0.0174	5.184	<0.05
1/256	0.8204 ± 0.0008	18.4360	<0.05	0.8708 ± 0.0183	1.3440	<0.05	0.9159 ± 0.0075	3.672	<0.05

This experiment investigated the inhibitory effect of Honghe FuJie lotion on the formation of *C. albicans* biofilm in three stages (early stage 8 hours, medium term 24 hours and maturation stage 48 hours). The results in Table 2 indicated that the Honghe Fujie lotion has a good inhibitory effect on the various stages of *C. albicans* biofilm formation

3.3. Inhibition of C. albicans Bud Tube Growth by Honghe Fujie Lotion

The results showed that the Honghe Fujie lotion had an inhibitory effect on the germination of *C. albicans* germ tubes, and the inhibitory rate increased in a concentration dependent manner (Figure 2).

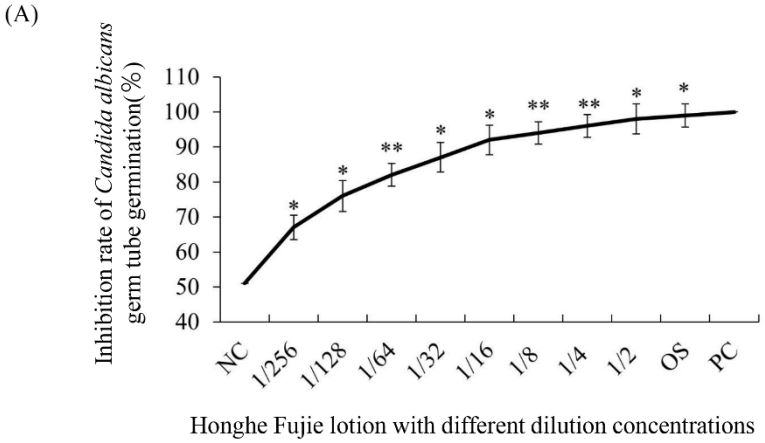


Figure 2. Inhibition effect of Honghe Fujie lotion on the germination of C. albicans germ tubes

3.4. Morphological Changes of C. albicans before and after Administration

Observing the microscopic morphology of Honghe Fujie lotion before and after administration. The cell wall of *C. albican*s without administration was round and smooth, and the boundary of the cell membrane was smooth and clear (Figure 3A). After 4 hours of treatment, the cell membrane and cell wall structure of *C. albicans* showed damage (Figure 3B).

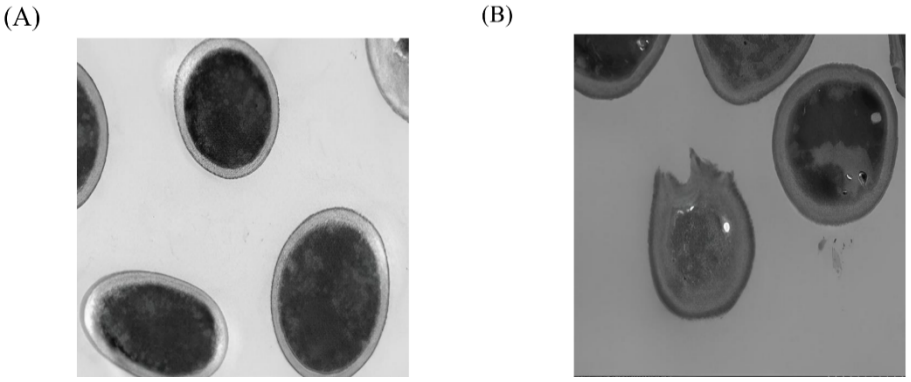


Figure 3. Microstructure of C. albicans before and after administration.

3.5. Effect of Honghe Fujie Lotion on the Growth of C. albicans in the Vagina of VVC Model Rats

On the eighth day after treating the vagina of rats with Honghe Fujie lotion, 5 uL of vaginal flushing solution was taken out and subjected to plate culture. The results showed that the number of bacterial colonies in the vagina of the rats in the Honghe Fujie lotion group was significantly lower than that of the model control group, and showed a certain degree of concentration dependence, the most obvious one was that the number of bacterial colonies in the vagina of rats in the positive control drug Amphotericin B group was higher than that in the vagina of rats treated with Honghe Fujie lotion (Figure 4).

3.6. The Effect of Honghe Fujie Lotion on Vaginal Pathological Tissue of VVC Model Rats

The pathological HE staining results of the vaginal tissue of VVC model rats were shown in Figure 5. The results showed that the epithelial structure of the vaginal mucosa in the normal group of rats was intacted and arranged in a regular manner, with no congestion, edema, or infiltration of inflammatory cells in the submucosal interstitium. The vaginal mucosal epithelial structure of the model control group rats was damaged and irregular, with high edema and congestion in the submucosa, and a large number of inflammatory cells infiltrating, indicating successful modeling. Comparing to the model group, the treatment group with Honghe Fujie lotion showed a dose-dependent reduction in vaginal mucosal damage in VVC model rats, hindered the infiltration and diffusion of inflammatory cells, and exhibited similar to the positive control drug Amphotericin B group.

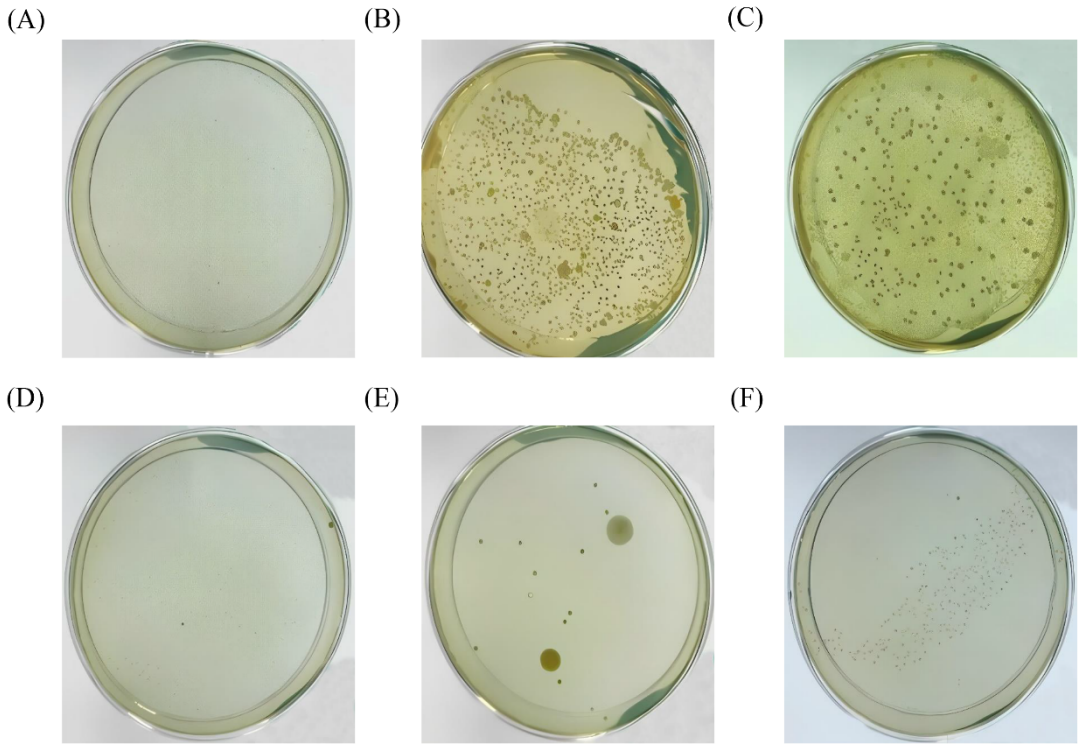


Figure 4. Inhibition of the growth of C. albicans in the vagina of VVC model rats with Honghe Fujie lotion. (A) Normal group. (B) Model group. (C) Amphotericin B group. (D-F) Diluting the groups 5 times, 10times and 20 times with Honghe Fujie lotion

4. Discussion

Multiple microbial communities participate in the balance of vaginal microbiota, in clinical research, the status of vaginal microbiota is mainly judged based on five indicators: dominant species of microbiota, density of microbiota, diversity of microbiota, host inflammatory response and pathogenic bacteria. This can to some extent reflect the relationship between microbiota evolution and diseases [10]. The first step in the formation of microbial biofilms is adhesion, which is also the most important link in biofilm formation. The prerequisite for causing microbial disease is that *C. albicans* adhere to the surface of host cells [11]. C. *albicans* germ tubes were non walled tubular structures that extend during germination. The formation of germ tubes was an important link in *C. albicans* adhesion, which helps to enhance its pathogenicity [12].

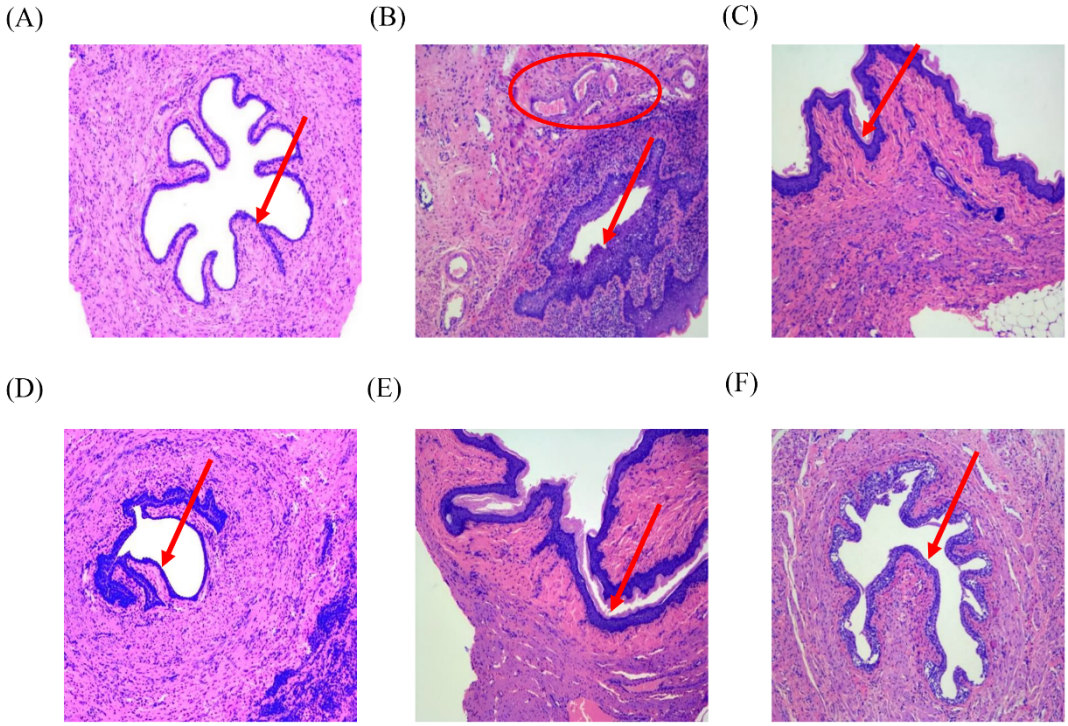


Figure 5. Honghe Fujie lotion improves inflammatory cell infiltration in vaginal tissues of VVC model rats (×100). (A) Normal group. (B) Model group. (C) Amphotericin B group. (D-F) Diluting the groups 5 times, 10times and 20 times with Honghe Fujie lotion

The experimental results indicated that Honghe Fujie lotion has a significant inhibitory effect on the formation of *C. albicans* germ tubes and biofilms. It was speculated that the Honghe Fujie lotion may inhibit the formation of *C. albicans* germ tubes, thereby reducing the adhesion of *C. albicans* and affected the formation of their biofilms, thereby producing antibacterial effects. The results indicated that Honghe Fujie lotion inhibited the formation of *C. albicans* germ tubes, suggested that its adhesion was a pathway for Honghe Fujie lotion to inhibit the growth of *C. albicans*.

The microstructure observation results before and after administration indicated that Honghe Fujie lotion can damage the cell wall and membrane structure of *C. albicans*. It was speculated that Honghe Fujie lotion may exert its inhibitory effect on *C. albicans* by destroying the cell wall and membrane structure.

The results of in vivo anti VCC experiments indicated that Honghe Fujie lotion can significantly reduce the formation of *C. albicans* colony in the vagina of VVC model rats, protected the vaginal mucosal tissue of rats, hindered the infiltration and diffusion of inflammatory cells, and improved the vaginal infection status of VVC model rats.

Honghe Fujie lotion can inhibit the growth and reproduction of *C. albicans* to a certain extent in the body, and can also act to inhibit bacterial growth and reproduction in vitro. In summary, the treatment of mycotic vaginitis with Honghe Fujie lotion can significantly improved patients' clinical symptoms, significantly improved clinical efficacy, and enhanced local vaginal immune function in a relatively short period of time. This experiment further improved the basic research on the antibacterial and anti-inflammatory effects of traditional Chinese patent medicines Honghe Fujie lotion, and provided a research basis for the clinical drug safety evaluation of Honghe Fujie lotion. Further exploration and research were needed on the key anti-inflammatory and antibacterial active ingredients in the experiment.

5. Conclusion

Honghe Fujie lotion can significantly reduce the formation of *Candida albicans* in the vagina of VVC model rats, protected the vaginal mucosal tissue of rats, hindered the infiltration and diffusion of inflammatory cells, and improved the vaginal infection status of VVC model rats.

Acknowledgments

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