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## Abstract

**Background:** *Rhizoma Coptidis* has been used as antibiotics in traditional Chinese medicine practice for many years. Here, we examined the effect of *Rhizoma Coptidis* extract on the growth of *C. albicans*.

**Materials and Methods:** The antifungal effects of *Coptidis Rhizoma* extract was examined by time-kill assay, transmission electron microscope (TEM) and scanning electron microscope (SEM) observations. The anti-inflammatory effect of the *Rhizoma Coptidis* extract was examined in mouse model of *Candida vaginitis*.

**Results:** We found that the MIC of *Coptidis Rhizoma* extract was 3.125 mg/mL. The time-kill assay suggested that *Rhizoma Coptidis* extract could inhibit the growth of *C. albicans*. The SEM and TEM observations showed notable changes of cell wall and cytoplasmic content of the *C. albicans* and complete collapse of yeast cell exposed to *Rhizoma Coptidis* extract. Our *in vivo* antifungal activity studies in mouse model of *Candidal vaginitis* showed the reduction of inflammatory reaction was found after *Rhizoma Coptidis* extract treatment. In addition, the COX-2 expression was decreased in mice treated with *Coptidis Rhizoma* extract as compared with the control mice.

**Conclusion:** Our results indicate that *Coptidis Rhizoma* extract is a promising candidate in *candidal vaginitis* treatment in the future.

**Key Words:** Antifungal activity, *Coptidis Rhizoma*, *C. albicans*

**Abbreviations:** TEM: transmission electron microscope; SEM: scanning electron microscope; COX-2: Cyclooxygenase-2; MIC: The Minimum Inhibitory Concentration; RPMI 1640: Roswell Park Memorial Institute 1640; MOPS: morpholinepropanesulfonic acid; *C. albicans*: *Candida albicans*

## Introduction

Vaginitis is the most common gynecologic condition. Bacterial vaginosis, trichomoniasis, and genital candidiasis account for 90% of all cases in reproductive age women (Mascarenhas et al., 2012). The patient typically has symptoms of vaginitis such as discharge, itching, burning, and possibly pain (De Seta et al., 2014). *Candida* vaginal colonization is thought to occur in 10% to 55% of healthy, asymptomatic women (Solis-Arias et al., 2014). It is estimated that 75% of women will experience an episode of candidal vaginitis in their lifetimes (Rathod and Buffler, 2014). The current available antifungal agents for candidal vaginitis include fluconazole, Nystatin and Clotrimazole (Osser et al., 1991). However, these antimycotics are frequently toxic at therapeutic dosage. Meanwhile, it has been reported that some strains of important fungal pathogens are resistant to current available antifungal agents (Xie et al., 2014). Therefore, there is need for development of novel antifungal drugs.

Chinese herbal medicine has been used to treat a wide range of diseases in the past hundred years. The potential benefits of Chinese herbal medicine include relative safety and low cost, as compared with synthetic drugs (Ke et al., 2012). *Rhizoma coptidis*, the dried Rhizome of *Coptis chinensis* Franch, is known as Huang Lian and frequently found in traditional Chinese herbal formulae. It has been reported that *Rhizoma coptidis* exert a number of pharmacological effects including antihypertensive (Tsai et al., 2008), antibacterial (Kong et al., 2009), and anti-oxidative (Jung et al., 2009) and anti-inflammatory effects (Kim et al., 2010). However, the exact mechanisms of action of *Rhizoma coptidis* have not been fully elucidated.

In the present study, we examined the anticandidal activity of *Coptidis rhizoma* extract both *in vitro* and *in vivo*. *Rhizoma coptidis* extract exhibited inhibitory effect on the growth of *C. albicans*. And the morphology changes were observed by SEM and TEM after *Coptidis rhizoma* extract treatment. The reduction of inflammatory reaction in mouse model of *Candidal vaginitis* was found after *Rhizoma coptidis* extract treatment. Interestingly, the COX-2 expression was decreased in mice treated with *Coptidis rhizoma* extract. Our findings indicate that *Coptidis rhizoma* extract serves as a potential candidate in candidal vaginitis treatment.

## Materials and Methods

### Microorganism

*C. albicans* ATCC 90028 purchased from American Type Culture Collection was used in this study. The cells were grown in Sabouraud dextrose broth (Zhaorui Bio Technologies, Shanghai), then inoculated onto Sabouraud dextrose agar (Zhaorui Bio Technologies, Shanghai) plates for 24 h at 30 °C. The cells were harvested by centrifugation followed by washing twice in phosphate-buffered saline. The cell pellet was suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, USA) supplemented with morpholinepropanesulfonic acid (MOPS).

### Preparation of *Coptidis Rhizoma* Extract

500 g of sliced *Coptidis rhizoma* was extracted with 500 mL of 70% ethanol by reflux at 95 °C for 3 h. Ethanol was removed under vacuum and the resulting aqueous concentrate was lyophilized to a powder.

### The Antifungal Activity of *Coptidis rhizoma* Extract Against *C. albicans*

The Minimum Inhibitory Concentration (MIC) of *Coptidis rhizoma* extract was determined using the broth micro-dilution method described in the guidelines of the National Committee for Clinical Laboratory Standards M27-A2 document (Uchida et al., 2006). Briefly, twofold final concentration serial dilutions of *Coptidis rhizoma* extract were prepared in RPMI 1640 medium ranging from 0.391 mg/mL to 25 mg/mL and added to each well of a sterile 96-well plate containing 100 µL of *C. albicans* suspension ( $2 \times 10^7$  cells/mL). Three wells containing fungal suspension with no drug and three wells containing only media were included in this experiment. Then, the 96-well plate was incubated at 30°C for 48 h and then the MIC value was determined both by direct observation and by determination of the number of CFUs. These experiments were performed three-times in triplicate.

### Time-Kill Curve Procedures

The time kill tests were conducted as described before (Klepser et al., 1998). Briefly, 500 µL of *C. albicans* suspension ( $2 \times 10^7$  cells/mL) was added to RPMI 1640 medium supplemented with MOPS. *Coptidis rhizoma* extract was added to individual cultures resulting concentrations equal to 0.5; 1 and 2 times the MIC. The test mixtures were incubated at 35°C with constant rotation. The growth of *C. albicans* was measured every 4 h for 48 h continuously, 100 µL of mixture were removed from each test suspension and the optical density at 540 nm was measured using UV-Visible spectrophotometer (UV-9100, Beifen-Ruili Analytical Instrument Co., China). All time-kill curve studies were conducted in triplicate.

### Transmission Electron Microscope (TEM) Observation

500 µL of *C. albicans* suspension ( $2 \times 10^7$  cells/mL) was inoculated on Sabouraud dextrose agar plate and then incubated at 35 °C for 6 h. *Coptidis rhizoma* extract was dropped onto inoculated agar and further incubated at the same condition. The *C. albicans* cells without treatment with extract were used as a control. The TEM analyses were performed on samples which were harvested at same time point (24, 48 and 72 hours) and fixed in McDowell-Trump fixative prepared in 0.1 M phosphate solution (pH 7.2) for 24 hours at 4 °C, followed by washing twice with the same buffer. The samples were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h at 4 °C. After dehydration with 50%, 75%, 95% and 100% ethanol, the samples were embedded in Spurr's resin. The ultra-thin sections of the cells were stained with 2% uranyl acetate and lead citrate and observed under a Transmission Electron Microscope (LIBRA 120, ZEISS).

### Scanning Electron Microscopy (SEM) Observation

*C. albicans* cells were prepared as described in TEM section. 500 µL of *C. albicans* suspension ( $2 \times 10^7$  cells/mL) was inoculated on Sabouraud dextrose agar plate and then incubated at 35 °C for 6 h. *Coptidis rhizoma* extract was dropped onto inoculated agar and further incubated at the same condition. The *C. albicans* cells without treatment with extract were used as a control. A small block of yeast containing agar was withdrawn from plate at 24 h and fixed for scanning. SEM observations were made under the following condition: EHT = 10.00 kV, Current = 0.12 nA, WD = 15 mm.

### Animals and Treatment

Thirty 7-week old female ICR mice were purchased from Beijing laboratory animal center in China. All procedures involving animals were carried out according to the international standards of animal care guidelines and were approved by the local Care of Experimental Animals Committee. Mice were randomly divided into two groups. 15 mice per experimental or control group were used. Three days prior to infection, mice were given 100 µL of sesame oil containing 0.5 mg of β-estradiol subcutaneously to induce pseudoestrus. On day 0, mice were anaesthetized with ketamine hydrochloride, then 20 µL of the inoculum suspension ( $2.5 \times 10^6$ /mL) in RPMI-1640 medium was injected into the vaginal lumen using pipette tip. Prior to treatment, the preliminary experiments were performed to assure infection was evenly distributed between groups. Vaginas were swabbed 48 hours later and each swab was placed in sterile PBS and serial 10-fold diluted, then 50 µL was placed onto Sabouraud dextrose agar plates with chloramphenicol to quantify the CFU/mL. *Coptidis rhizoma* extract was suspended directly in RPMI 1640 medium and was given by intra-vaginal instillation (25mg/kg) for six consecutive days. RPMI 1640 medium was given to mice in control group.

### Vaginal Lavages

On day 2, 6 and 10, mice were anaesthetized with ketamine hydrochloride and lavaged the vaginal lumen by introducing 100 µL of sterile PBS with repeated aspiration and agitation with a pipette tip. 10 µL of the lavage fluid was transferred onto a glass slide and observed directly under light microscopy. The left lavage fluid was serial 10-fold diluted and 50 µL was placed onto Sabouraud dextrose agar plates with chloramphenicol to quantify the CFU/mL after incubation at 35°C for 48 h.

### Immunohistochemical Analysis

Mice were sacrificed by CO<sub>2</sub> exposure. The vaginas were removed and fixed in 10% neutral formalin buffer for 48 h, dehydrated, and wax embedded. Paraffin-embedded tissues were sliced as 4- $\mu$ m sections and stained with hematoxylin and eosin (H&E) for morphological analysis. For immunohistochemical staining, the sections were incubated with COX-2 primary antibody (cell signaling, USA), followed by incubation with secondary antibody (1:2000) conjugated to streptavidin peroxidase. Color was developed with diaminobenzidine peroxidase substrate solution, and the sections were counterstained with hematoxylin. The sections were examined by computer-assisted image analysis (DMR+Q550; Leica) and a microphotography system (Vanox; Olympus, Japan).

### Statistical Analysis

The results are expressed as means and standard deviations. Differences between groups were analyzed by Student's t-test using the SPSS program (version 13.0) for windows (SPSS, Chicago, IL, USA). Differences are considered statistically significant if  $p < 0.05$ .

## Results

### Drug susceptibility

To examine the effect of *Coptidis rhizoma* extract on growth of *C. albicans* and to determine its antifungal activity, the cells were grown in 96-well plates containing *Coptidis rhizoma* extract and the MIC was measured using the broth dilution method. We found that the MIC of *Coptidis rhizoma* extract was 3.125 mg/mL. Then time-kill assay was performed over a period of 48 hour with cells being exposed to 1/2, 1 or 2 x MIC of *Coptidis rhizoma* extract. As shown in Fig. 1, at 1/2 x MIC, we found that *Coptidis rhizoma* extract showed a dramatic inhibition on cell growth after 16 hours. At MIC and 2 x MIC, *Coptidis rhizoma* extract inhibited the cell growth after 12 hours. Our results indicate that *Coptidis rhizoma* extract might be a potential candidate in the treatment of candidosis.

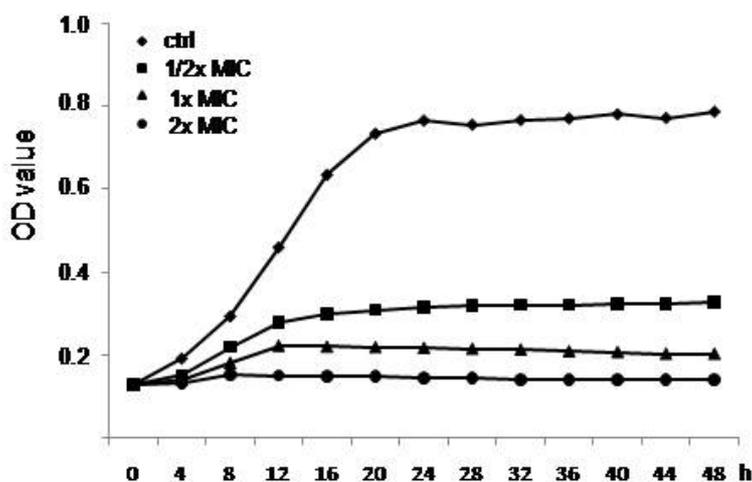
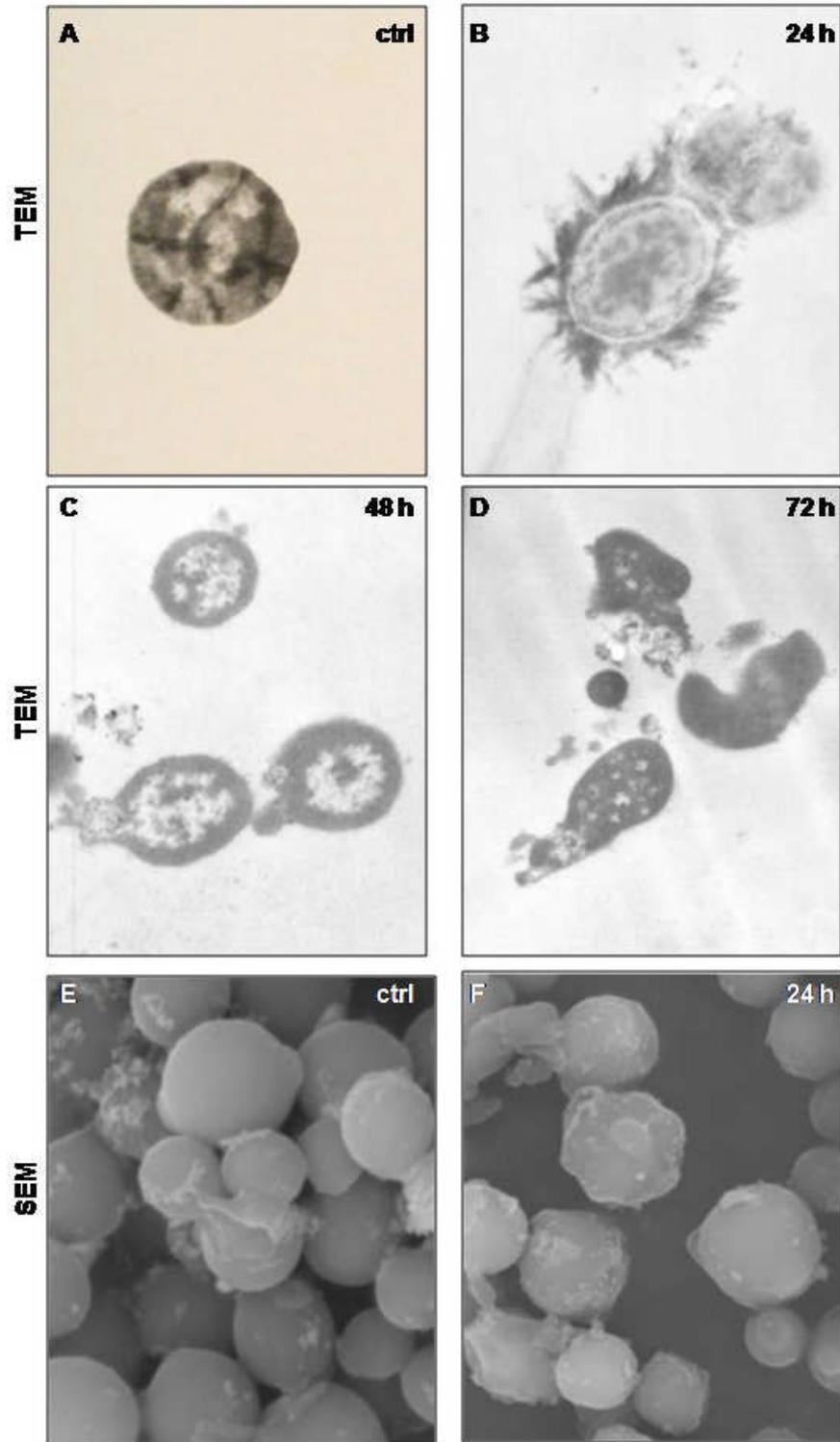


Figure 1: Time-kill curve of *Coptidis rhizoma* extract on *C. albicans* over a period of 48 hour.

### The Morphology Changes of *C. albicans* after *Coptidis rhizoma* Extract Treatment

To observe the morphology change of *C. albicans* after treated with *Coptidis rhizoma* extract, The TEM and SEM analyses were performed. The Fig. 2 showed the morphology changes of *C. albicans* after *Coptidis rhizoma* extract treatment by TEM (Fig. 2 A-D) and SEM (Fig. 2 E-F) analyses. Without treatment, the cytoplasm of *C. albicans* cells was homogeneous. The nucleus and mitochondria were surrounded by a defined cell membrane (Fig. 2 A). After 24 h of exposure to *Coptidis rhizoma* extract, the cell was very dense with the vesicles and membranous bodies positioned within the cell (Fig. 2 B). After 48 hours treatment, the cell wall was wrinkled. The decreased cytoplasmic volume was found and the cell membrane in-vaginated with notable structural disorganization within the cell cytoplasm (Fig. 2 C). After 72 hours of exposure, the inner organelles were completely decomposed and even cell membrane and wall were affected. Yeast cells were found collapsed which followed by an outflow of the cytoplasmic component (Fig. 2 D). The SEM observations showed that the control cells were regular and homogeneous, the cell walls were smooth and some cells are in budding stage. After 24 hours treatment, the well-defined wrinkling of cell wall in some cells was found. Some cells completely collapsed or cavitated, and the rough surfaces with holes were seen (Fig. 2 F).



**Figure 2:** The morphology changes of *C. albicans* after *Coptidis rhizoma* extract treatment. (A) TEM micrograph of a cross-section of untreated cell of *C. albicans* and extract-treated after 24 h (B), 48 h (C) and 72 h (D). Scanning electron microscope photomicrograph of the untreated (E) and extract-treated (F) cells of *C. albicans*.

### Quantification of Vaginal Fungal Burden and the Cellular Fractions of Vaginal Lavage Fluid

To examine the vaginal fungal burden, vaginal lavage fluid was collected at specific time points and cultured for CFU enumeration. As shown in Fig. 3, *C. albicans* infection was found in both control group and treatment group on day 2. However, after 6 days treatment with *Coptidis rhizoma* extract, *C. albicans* cells were significantly eliminated in treatment group. The *C. albicans* infection did not reoccur on day 11. We further determined the cellular fractions of vaginal lavage fluid at specific time points by wet-mount and pap smear. As shown in Fig. 4, the *C. albicans*, epithelial cells and neutrophils can be found in both control group (Fig. 4A) and treatment group (Fig. 4B) on day 2. However, after treatment, the number of yeast cells and neutrophils was dramatically decreased on day 6 (Fig. 4 E-F) and day 11 (Fig. 4 H-I) as compared with the control group (Fig. 4 D and G), which is consistent with the vaginal fungal burden results.

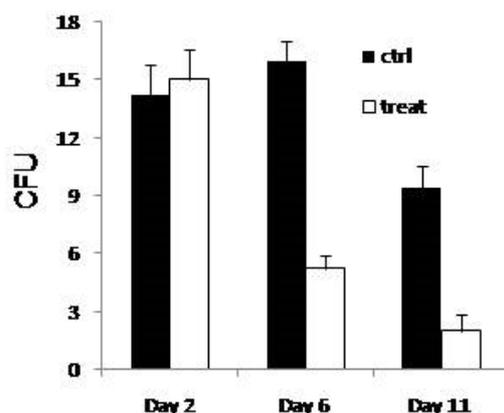


Figure 3: Quantification of vaginal fungal burden

### Histopathology

We further evaluated the inflammation after treatment by H&E staining on mice vagina mucosal surface. We found that infiltration of polymorphonuclear cells in the keratinized and deeper layers of the epithelium in control group (Fig. 5 A). PAS stain of a vaginal section from the same mouse showed infection with pseudohyphae and blastoconidia (Fig. 5 C). H&E stain of a vaginal section from treatment mouse showed a profound lack of neutrophils in both the keratinized and deeper layers of the epithelium (Fig. 5 B). Pseudohyphae and blastoconidia were not found in the same mouse by PAS stain in treatment group (Fig. 5 D). We further examined the COX-2 expression in mice vagina tissue after treatment by Immunohistochemical staining. As shown in Fig. 5F, we found that the COX-2 expression was decreased in mice treated with *Coptidis rhizoma* extract as compared with the control mice (Fig. 5 E).

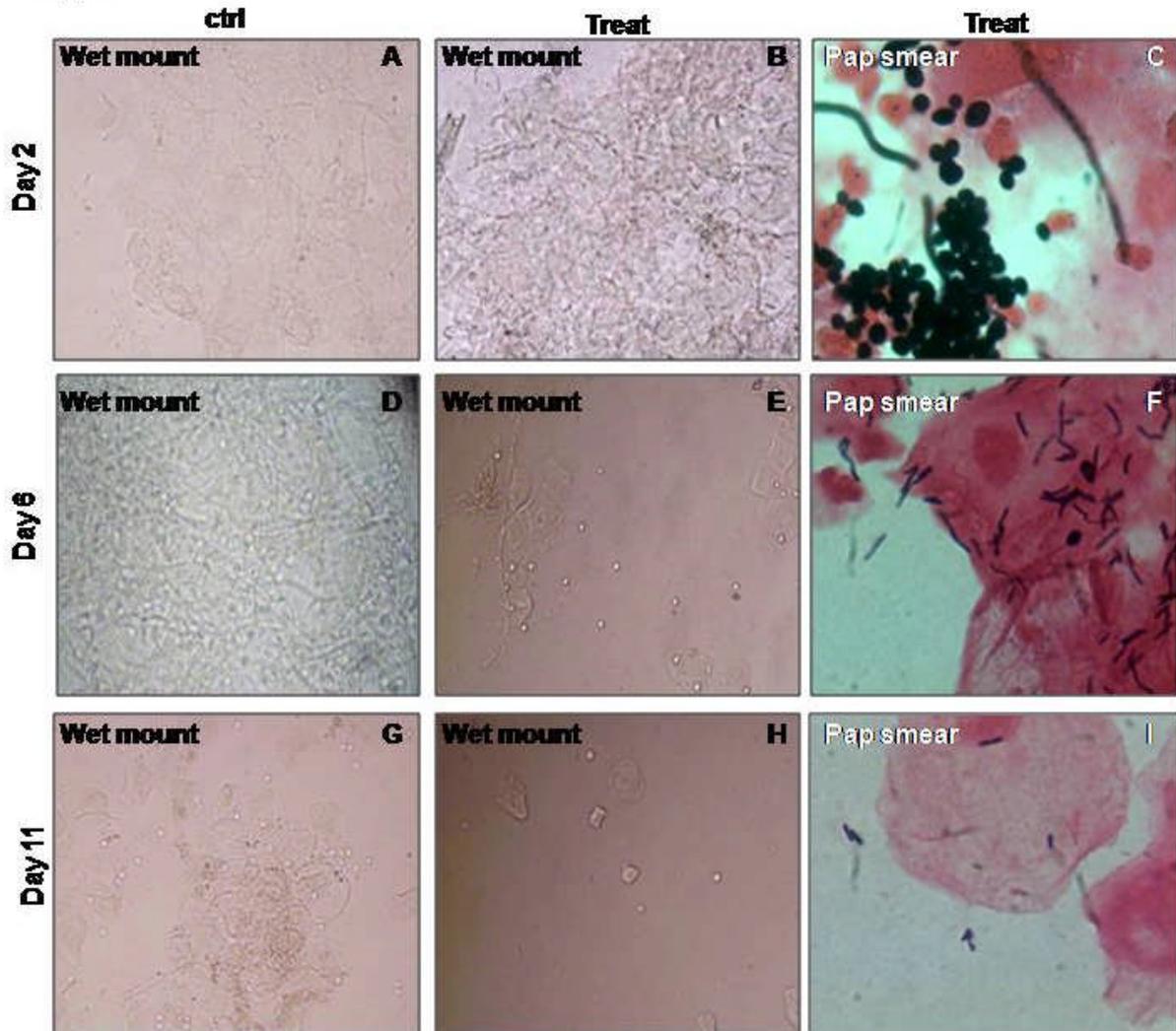
### Discussion

*Coptidis rhizoma* is known as the antibiotics in traditional Chinese medicine and has been used for thousand years in China (Luo et al., 2013). Kim et al found that *Rhizoma coptidi* exhibited anti-inflammatory effects in animal models of acute and chronic inflammation by inhibiting the production of NO and PGE<sub>2</sub> (Kim et al., 2008). Another team identified the anti-inflammatory effects of *Rhizoma coptidis* extract by inhibiting the expression of various proinflammatory cytokines and cell surface molecules involved in inflammatory responses at the transcriptional level (Kim et al., 2010). The anti-bacterial activities of *Rhizoma coptidis* have been reported (Kong et al., 2009). Recently, the antifungal activities of *Rhizoma coptidis* have attracted more attention (Hu et al., 2000; Seneviratne et al., 2008). In our study, *Rhizoma coptidis* extracts exhibited a favorable anti-*C. albicans* activity with a MIC value of 3.125 mg/mL. We further examined the ability of *Rhizoma coptidis* extract to inhibit *C. albicans* growth in vitro by time-kill assay. We found that *Coptidis rhizoma* extract showed a dramatic inhibition on cell growth after 16 hours even at 1/2 x MIC. At higher concentration of *Rhizoma coptidis* extract, the cell growth was inhibited after only 8 hours treatment. Then, we evaluated the morphology changes of *C. albicans* after *Coptidis rhizoma* extract treatment using SEM and TEM methods. The SEM observations showed that the yeast cells treated with *Coptidis rhizoma* extract decreased in size, changed in shape of cell walls. TEM observation showed damaged cell membranes, irregular cell walls and dense cytoplasm. The antifungal activities against *C. albicans* were further confirmed by animal model study. 7-week old female ICR mice were infected with *C. albicans* and treated with *Coptidis rhizoma* extract. We found that *C. albicans* cells were eliminated significantly in vaginal lavage fluid in treatment group. Wet-mount and pap smear showed that yeast cells and neutrophils was dramatically decreased by the *Coptidis rhizoma* extract treatment. Histological analysis showed that the inflammatory reaction was decreased in mice vagina tissues after treatment.

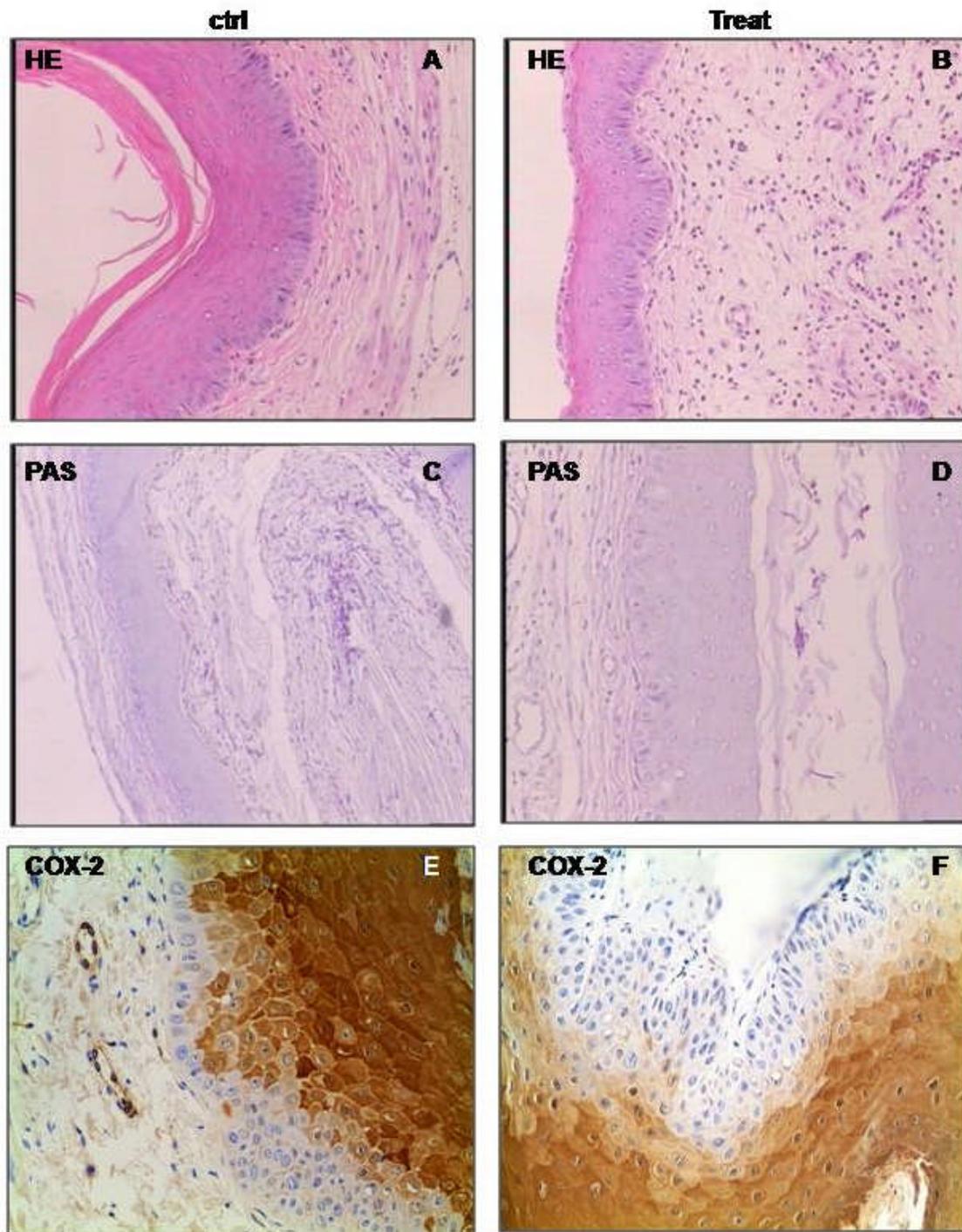
Cyclooxygenase-2 (COX-2) plays important roles in inflammation and in proliferative diseases, such as cancer (Ricciotti and FitzGerald, 2011). COX-2 can be induced in response to pro-inflammatory stimuli, cytokines, and mitogens. Some herbal medicines, such as Tumeric (Lantz et al., 2005), Ginger (van Breemen et al., 2011) and Boswellia (Cao et al., 2010), have showed anti-inflammatory properties through their role as botanical

COX-2 inhibitors. By immunohistochemical staining, we found the COX-2 expression was decreased in mice treated with *Coptidis rhizoma* extract as compared with the control mice. These results indicate the anti-inflammatory effect of *Coptidis rhizoma* extract in animal model.

In summary, *Coptidis rhizoma* extract displayed significant effect on inhibition of the growth of *C. albicans* both in vitro and in vivo. Our studies indicate that *Coptidis rhizoma* extract is a potential candidate in candidal vaginitis treatment in the future.



**Figure 4:** Cellular fractions of vaginal lavage fluid from inoculated mice. Wet-mount of vaginal lavage fluid in control group (A) and treatment group (B) on day 2. Pap smear preparations of vaginal lavage samples collected 2 days post-inoculation (C) from treated mice. Wet-mount of vaginal lavage fluid in control group (D) and treatment group (E) on day 6. Pap smear preparations of vaginal lavage samples collected 6 days post-inoculation (F) from treated mice. Wet-mount of vaginal lavage fluid in control group (G) and treatment group (H) on day 11. Pap smear preparations of vaginal lavage samples collected 11 days post-inoculation (I) from treated mice.



**Figure 5:** Vaginal histology analysis of the mouse model of candidal vaginitis. (A) H&E stain of a vaginal section in control group, (B) H&E stain of a vaginal section in treatment group, (C) PAS stain of a vaginal section in control group, (D) PAS stain of a vaginal section in treatment group, (E) Immunohistochemical staining of COX-2 in vaginal tissues in control group, (F) Immunohistochemical staining of COX-2 in vaginal tissues in treatment group.

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**Conflict of Interest :** All authors declare that there are no conflicts of interest.

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