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ANTIFUNGAL ACTIVITY OF ETHANOL EXTRACT OF *Phellodendron amurense* AND *Cochinchina mormodica* AGAINST *Microsporium canis*-INDUCED DERMATITIS IN RABBITS.

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## ANTIFUNGAL ACTIVITY OF ETHANOL EXTRACT OF *PHELLODENDRON AMURENSE* AND *COCHINCHINA MORMODICA* AGAINST *MICROSPORUM CANIS*-INDUCED DERMATITIS IN RABBITS

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### ABSTRACT

This study was performed to evaluate the antifungal effects of ethanol extraction of *Phellodendron amurense* (PAEE) or *Cochinchina momordica* seed (CE) or their combination (PCEE) against *Microsporium canis* (*M. canis*). The minimum inhibiting concentrations (MIC) of PAEE, CE and PCEE against *M. canis* were assessed using a micro dilution test. In addition, transmission electronic microscopy (TEM) was performed to observe their effects on cell ultrastructure. Differential expression of PCEE for *M. canis* inhibitory effect of NADH and FSH genes was also performed. Clinical evaluation was performed using an *in vivo* antifungal assay. TEM showed that 50, 100, 200, or 400µg/mL of PCEE destroyed the cell membrane or organelles of *M. canis*. The clinical results showed that PCEE was better than PAEE or CE. Significant difference was observed in FSH expression in PCEE 100 µg group as compared to PCEE 25 µg, clotrimazole 50, 25 µg and control groups (DMSO group) ( $P < 0.05$ ). The expression of FSH decreased with increasing concentration of PCEE. PCEE has significant antifungal activity and could be used to treat *M. canis* infection in rabbits.

**Keywords:** Chinese herbal compound; *Phellodendron amurense*; *Cochinchina momordica* seed; *Microsporium canis*; antifungal mechanism

### INTRODUCTION

Dermatophytes are pathogenic fungi that can invade the keratinized structure and infect skin, hair and nails of humans and animals. The species *Microsporium canis* (*M. canis*) is found in humans and animals, and is zoonotic in nature. *M. canis* are a major cause of dermatophytosis in pets and rabbits. A total of 21 isolates belonging to *M. canis* were collected from rabbits with or without skin lesions (Cafarchia et al., 2012).

Rabbit dermatomycosis is a type of highly infectious zoonotic contact dermatitis. The main clinical signs of the disease are dandruff, hair loss, exudation, crusting, folliculitis, itching and other symptoms (Zhang et al., 2009). The most common causative agents are *Trichophyton mentagrophytes* and *Microsporium* spp. (Lu and Chu, 2011). Various drugs, including azoles, propylene amine, ring ketone amine, amorolfine are available, but the overall effect is not stable, requires repeated usage, long-term treatment, is easy to relapse and has high cost (Xue et al., 2002). The interest in natural medicine has increased remarkably in recent years as a result of side effects of conventional drugs, as well as the emergence of resistance against available drugs. The bark from the Phellodendron tree has been used as traditional Chinese medicine for thousands of years. The species *Phellodendron amurense* (*P. amurense*) is widely used to treat gastroenteritis, abdominal pain, diarrhea and various inflammatory diseases, including arthritis and dermatophytosis (Xiao et al., 2015). The seeds of *Cochinchina momordica* (*C. momordica*) have proven to be very effective for curing dermatophytosis in humans (Cao et al., 2004). In this study, the ethanol extract of *P. amurense* mixed with *C. momordica* seed was used to treat dermatosis caused by *M. canis* in rabbits. Antifungal mechanism *in vitro* was also studied.

## MATERIALS AND METHODS

### Preparation of PAEE, CE and PCEE

Two hundred gram of *P. amurense* or *C. momordica* (*P. amurense* + *C. momordica* each 100g) were finely ground using an electric blender and extracted with 75% ethanol at 80°C twice under reflux for 1 h. After filtration and centrifugation (1700×g, 30 min), the combined solution was concentrated under reduced pressure with a rotary evaporator at 65°C until solid PAEE (ethonal extraction of *P. amurense*) (27.89g), CE (ethonal extraction of *C. momordica*) (21.25g) and PCEE(ethonal extraction of *P. amurense* + *C. momordica*) (25.6g) was obtained.

### *Microsporium canis* strains and experimental rabbits

The eumycete isolated from dermatopathic rabbits was obtained from Jiaxing district. The collection was grown in TSA plate for 96h at 28°C, moisture 60%. Eighteen 40-day-old healthy New Zealand rabbits were purchased from Yangdu Warren in Zhejiang Academy of Agricultural Sciences.

### *In vitro* antifungal effect of PAEE, CE and PCEE

The eumycete was grown on tryptic soy agar (TSA) plates at 28 °C with 60% humidity for four days. The minimum inhibitory concentration (MIC) of PCEE for *M. canis* was determined using a micro dilution test (Liu et al., 1997). PAEE, CE, PCEE and clotrimazole were dissolved in RPMI 1640 medium (Gibco) containing 10% DMSO. One hundred µl of RPMI was added to each well of a 96-well plate, then a final concentration of PAEE, CE, PCEE (2.5mg/ml) or clotrimazole (1.3mg/ml) was added to the first well. Next, 100 µl of the mixture was taken from the first well and added to the second well, mixed thoroughly, and then 100 µl of the mixture was taken and added to the next well. The same method was repeated until the twelfth well. After that, 100µl of eumycete was added to each well. The plates were incubated at 28 °C with 60% humidity. Seven days later, OD 450 nm was read in SpectraMaxM5 microplate reader enzyme mark instrument. MIC was calculated based on the method of Liu et al. (1997). Each test was repeated three times.

### *In vivo* antifungal assay

Experimental infection was performed as previously reported (Xiao et al, 2015). PAEE (0.05g/ml), CE (0.05g/ml), PCEE (0.05g/ml), clotrimazole (0.05g/ml) or distilled water were applied topically 24 h after infection and continued for three days as shown in Table 1. The lesions were evaluated as previously described (Ghannoum et al., 2008). The Bioethics Committee of the Zhejiang Academy of Agricultural Science approved this experiment, and the experimental procedures strictly complied with accepted international rules and regulations. The percent efficacy was calculated according to the formula described by Mikaeili et al. (2011). On the last day of experiment, three rabbits were selected from each group, and sterile forceps were used to remove the attack site hair, dander, separated into the TSA plate, placed in the mold box and cultured for five days. The separation results were observed.

**Table 1.** The characteristics of PCEE-treated and other groups in a rabbit model of dermatophytosis

| Group        | No. rabbits | Challenge (days) | Treatment       |        |   |
|--------------|-------------|------------------|-----------------|--------|---|
|              |             |                  | Application     | Day(s) | Quantity                                      |
| PAEE         | 6           | 1, 2, 3          | PAEE (0.05g)    | 4,5,6  | Animals received 1 ml of 0.05g/ml PAEE        |
| CE           | 6           | 1, 2, 3          | CE (0.05g)      | 4,5,6  | Animals received 1 ml of 0.05g/ml CE          |
| PCEE         | 6           | 1, 2, 3          | PCEE (0.05g)    | 4,5,6  | Animals received 1 ml of 0.05g/ml PCEE        |
| Clotrimazole | 6           | 1, 2, 3          | Clotrimazole    | 4,5,6  | Animals received 1ml of clotrimazole 0.05g/ml |
| Control      | 6           | 1, 2, 3          | Distilled water | 4,5,6  | Animals received 1 ml of distilled water      |

### Ultrastructural analysis by transmission electron microscopy (TEM)

TEM was used to observe the effect of extract on cellular ultra-structure as previously described (Basma et al., 2011). One ml of *M. canis* cells ( $5 \times 10^9$  CFU/ml) was treated with PCEE (50,100,200,400µg/mL) or clotrimazole (50µg/mL) for 5 h. Sections were observed with a JEM-1230 TEM (JEOL, Japan).

### Differential expression of PCEE for *Microsporium canis* inhibitory effect of NADH and FSH genes

One ml of *M. canis* cells ( $5 \times 10^9$  CFU/ml) was treated with 100, 50, 25 or 0  $\mu\text{g}$  of PCEE or clotrimazole 100, 50  $\mu\text{g}$  for 5 h. Each group had six samples. RNA was extracted (Zhang et al., 2011) using the Trizol method. The primers of NADH (Nicotinamide adenine dinucleotide dehydrogenase) and FSH Serine protease) were designed according to the gene sequence of *M. canis* listed in Gene Bank (Table 2). The 18S ribosomal gene was used as the control. Relative quantification between samples was achieved by the  $2^{-\Delta\Delta\text{CT}}$  method (Livak and Schmittgen, 2001). The procedures were as the following: 95°C for 30min, [95°C for 5 sec, 60 °C for 30 sec, 95 °C for 15sec] from step 2 to step 4 were repeated 40 cycles, 60°C for 1min, 95 °C for for 15s. Each reaction was analyzed at least trice. The primers for NADH and FSH genes were designed according to Zhang et al. (2011). Relative quantification between samples was achieved by the  $2^{-\Delta\Delta\text{CT}}$  method (Livak and Schmittgen, 2001).

**Table 2 :** The primer of genes used in real-time PCR

| Gene symbol | Primer | Primer sequence, 5' → 3'        | Length, bp |
|-------------|--------|---------------------------------|------------|
| NADH        | F      | CCTGCTTTACTTATAGTAGCTTTTGTTACAA | 114 bp     |
|             | R      | AAATGCTTGGAGTAAACCATAATAACC     |            |
| FSH         | F      | TGCTGAGAAGAGACAGGCAAAC          | 73 bp      |
|             | R      | GCTGTCATATTTCTACGACAACAA        |            |
| 18S         | F      | TGGTGCATGGCCGTTCTTA             | 65 bp      |
|             | R      | GGTCTCGTTCGTTATCGCAATT          |            |

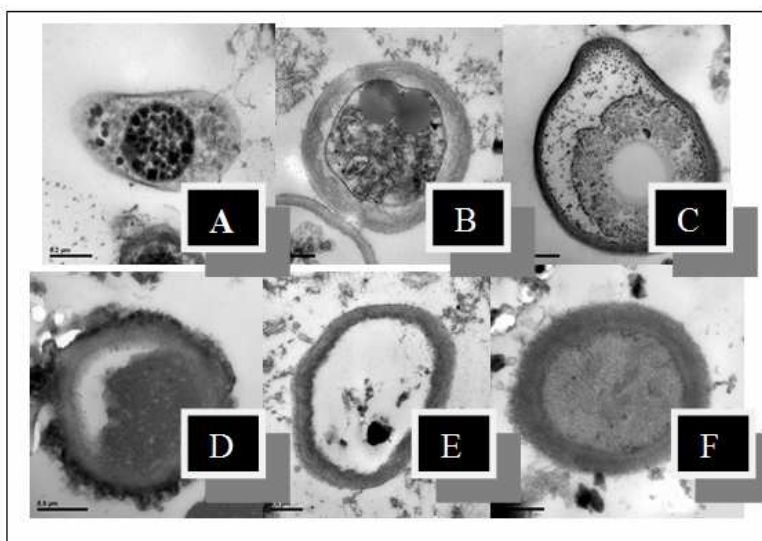
### Statistical Analysis

Comparison of means for *in vivo* antifungal activity was performed using one-way analysis of variance (ANOVA) and Tukey HSD test.  $P < 0.05$  was considered to be statistically significant. Data were expressed as mean  $\pm$  SD.

## RESULTS

### *In vitro* fungistatic effect of PAEE, CE and PCEE

According to the results of OD 450, MIC90 of PAEE, CE and PCEE was 62  $\mu\text{g}/\text{ml}$ , 516 $\mu\text{g}/\text{ml}$  and 16.67  $\mu\text{g}/\text{ml}$ , respectively, and MIC90 of clotrimazole was 10  $\mu\text{g}/\text{ml}$ . *M. canis* under different concentrations of PCEE for five hours showed different degrees of morphological changes (Figure 1).



**Figure 1.** Electron microscopic observation of the morphology of *Microsporium canis* under different doses of PCEE or clotrimazole.

A, B, D, E, delegate 50, 100, 200, 400  $\mu\text{g}/\text{mL}$  PCEE; C, F delegate clotrimazole ( 50  $\mu\text{g}/\text{mL}$  ) or negative(DMSO)

The nuclei color of PCEE 50  $\mu\text{g}/\text{ml}$  group was black; cell membrane of PCEE 100  $\mu\text{g}/\text{ml}$  group showed cell-wall separation; PCEE 200  $\mu\text{g}/\text{ml}$  group showed vacuoles; PCEE 400  $\mu\text{g}/\text{ml}$  group cell organelles showed concentrated cleavage. Clotrimazole 50  $\mu\text{g}/\text{ml}$  group showed morphological changes in cell organelles and the

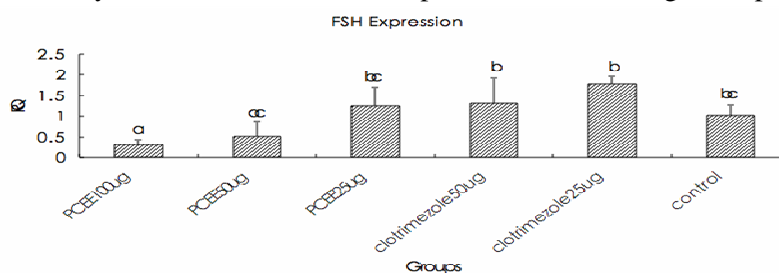
nucleus. Negative control was normal.

### Effect of PCEE on the expression of NADH and FSH genes

Inhibitory effects of PCEE on the expression of NADH and FSH genes of *M. canis* are shown in Figures 2 and 3. There was no significant difference in NADH gene expression. There was significant difference in FSH expression in PCEE 100 µg group as compared to PCEE 25 µg, clotrimazole 50, 25 µg, and control group ( $P < 0.05$ ). The expression of FSH decreased with increasing concentration of PCEE.



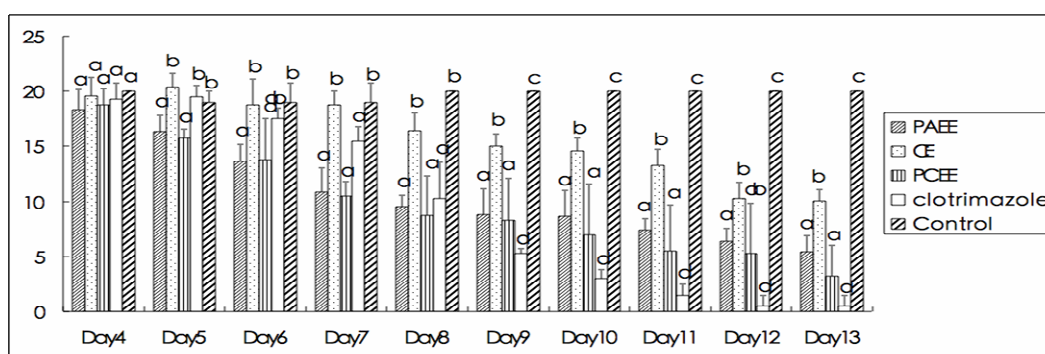
**Figure 2.** Inhibitory effect of PCEE on *Microsporium canis* NADH gene expression.



**Figure 3.** Inhibitory effect of PCEE on *Microsporium canis* FSH gene expression

### In vivo antifungal assay

The skin lesions of PAEE and PCEE groups started to subside from day 5, with significant recovery as compared to the control and clotrimazole groups. Significant increase ( $P < 0.05$ ) in recovery was observed in all treatment groups when compared with the negative control (Figure 4) from day 7, except the CE group. From day 9 onwards, recovery of clotrimazole group was significantly better than PAEE, CE or PCEE until day 13. Clotrimazole effect occurred later than PAEE or PCEE, with better efficacy at the end of the experiment. Although there were no significant differences between the PAEE and PCEE groups, PCEE seemed more effective than PAEE, perhaps due to the combination of two herbs.



**Figure 4.** Effect of PAEE, CE, PCEE, Clotrimazole and distilled water (control). Significant differences are shown with different alphabets at  $P < 0.01$ .

## DISCUSSION

In this study, MIC values of the combination of two herbal extracts (PCEE) for *M. canis* were similar to clotrimazole. The PCEE antifungal activity is similar to some synthetic antifungal medications. The MIC value of PAEE or CE was much higher than PCEE, which showed the combination effect of these two herbs. Electron microscopy showed that ( 50,100,200,400µg/mL ) of PCEE can have damaging effects

on spore cell membrane, nucleus and organelles, which inhibits the normal growth of fungi. *M. canis* infection and treatment test showed that in clinical practice, PCEE is effective for the treatment of rabbit dermatomycosis caused by *M. canis*. Skin PAS staining results showed the cuticle within few fungal distribution after PCEE treatment, while the control group had numerous fungi. The results of clinical experiment suggested that clotrimazole take time to exert its effects, but could cure better than PCEE at the end of the experiment. Although it worked better than PCEE, drug tolerance of synthetic antifungal medications will influence further treatment. Antifungal mechanisms of natural drugs remain unclear. FSH1 is a serine hydrolase that contains a Ser/His/Asp active site and belongs to a multifunctional alpha/beta hydrolase subfamily (Zhang et al., 2011). It plays an amidase role in endogenous signal factor in the regulation of growth and is elevated upon infection of the host skin. FSH gene was expressed in the 100 µg dose group, and its downward trend suggested that the virulence of *M. canis* declined with 100 µg dosage indicating that PCEE could reduce the infection ability of *M. canis* in rabbits.

## CONCLUSIONS

The Chinese herbal extract PCEE was effective in treating fungal skin disease caused by *Microsporum canis*.

## ACKNOWLEDGMENTS

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