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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 Microsporum 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; Microsporum 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 Microsporum 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 Microsporum 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.

*Microsporum canis* strains and experimental rabbits
The eumycete isolated from dermopathic 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 throughly, 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

<table>
<thead>
<tr>
<th>Group</th>
<th>No. rabbits</th>
<th>Challenge (days)</th>
<th>Treatment</th>
<th>Application</th>
<th>Day(s)</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAEE</td>
<td>6</td>
<td>1, 2, 3</td>
<td>PAEE (0.05g)</td>
<td>4, 5, 6</td>
<td></td>
<td>Animals received 1 ml of 0.05g/ml PAEE</td>
</tr>
<tr>
<td>CE</td>
<td>6</td>
<td>1, 2, 3</td>
<td>CE (0.05g)</td>
<td>4, 5, 6</td>
<td></td>
<td>Animals received 1 ml of 0.05g/ml CE</td>
</tr>
<tr>
<td>PCEE</td>
<td>6</td>
<td>1, 2, 3</td>
<td>PCEE (0.05g)</td>
<td>4, 5, 6</td>
<td></td>
<td>Animals received 1 ml of 0.05g/ml PCEE</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>6</td>
<td>1, 2, 3</td>
<td>Clotrimazole</td>
<td>4, 5, 6</td>
<td></td>
<td>Animals received 1 ml of clotrimazole 0.05g/ml</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>1, 2, 3</td>
<td>Distilled water</td>
<td>4, 5, 6</td>
<td></td>
<td>Animals received 1 ml of distilled water</td>
</tr>
</tbody>
</table>

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 (5×10⁹ 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 *Microsporum canis* inhibitory effect of NADH and FSH genes

One ml of *M. canis* cells (5×10^9 CFU/ml) was treated with 100, 50, 25 or 0 µg of PCEE or clotrimazole 100, 50 µg 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−⊿⊿CT method (Livak and Schmittgen, 2001). The procedures were as the following: 95℃ for 30min, [95℃ for 5 sec, 60℃ for 30 sec, 95℃ for 15sec] from step 2 to step 4 were repeated 40 cycles, 60℃ for 1min, 95℃ 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−⊿⊿CT method (Livak and Schmittgen, 2001).

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

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer</th>
<th>Primer sequence, 5′→3′</th>
<th>Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>F</td>
<td>CCTGCTTTACTTATAGTAGCTTTTGTTACAA</td>
<td>114 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>AAATGCTTGGAGTAACCAATAAACCA</td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>F</td>
<td>TGCTGAGAAGAGACAGGCAAAC</td>
<td>73 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GCTGTCAATTTCTACGACAACAA</td>
<td></td>
</tr>
<tr>
<td>18S</td>
<td>F</td>
<td>TGGTGCAATGGCGTTCTTTA</td>
<td>65 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GGTCCTCGTTCGTTATGCAATT</td>
<td></td>
</tr>
</tbody>
</table>

**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 ± 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 µg/ml, 516µg/ml and 16.67 µg/ml, respectively, and MIC90 of clotrimazole was 10 µg/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 *Microsporum canis* under different doses of PCEE or clotrimazole.](image)

A, B, D, E, delegate 50, 100, 200, 400 µg/mL PCEE; C, F delegate clotrimazole (50 µg/mL) or negative(DMSO)

The nuclei color of PCEE 50 µg/ml group was black; cell membrane of PCEE 100 µg/ml group showed cell-wall separation; PCEE 200 µg/ml group showed vacuoles; PCEE 400 µg/ml group cell organelles showed concentrated cleavage. Clotrimazole 50 µg/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](image)

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

![Figure 3](image)

**Figure 3.** Inhibitory effect of PCEE on Microsporum 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](image)

**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, 400 µg/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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**REFERENCES**


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