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RESEARCH ARTICLE

Antifungal Efficacy of *Phellodendron amurense* Ethanol Extract against *Trichophyton mentagrophytes* in Rabbits

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ABSTRACT

The bark of the *Phellodendron* tree has been used in traditional Chinese medicine for thousands of years. *Phellodendron amurense* is widely used to treat gastroenteritis, abdominal pain, diarrhea and various inflammatory diseases including arthritis and dermatophytosis. The present study was undertaken to evaluate the antifungal effects of *Phellodendron amurense* ethanol extract (PAEE) against *Trichophyton mentagrophytes in vivo* and *in vitro*. Quantitative analysis revealed that the level of total alkaloids in PAEE was 7.58±0.46 mg/mL. In the *in vitro* study, the minimal inhibitory concentration (MIC) of PAEE (0.03g/mL) and clotrimazole (0.02 mg/mL) were determined using 1.5% tryptic soya agar. The influence of different doses of PAEE on the growth of *T. mentagrophytes* was detected by dry weight determination. Moreover, transmission electronic microscopy was performed to observe the effect of PAEE on cell ultrastructure, and it showed that PAEE destroyed the cell membrane of *T. mentagrophytes*. Furthermore, the dermatophytosis infection model in rabbits with *T. mentagrophytes* was established for investigating the *in vivo* effect of PAEE at 10, 50 and 100% concentrations. The findings in all treatment groups demonstrated the inhibitory effects of PAEE against *T. mentagrophytes*. Therefore, we conclude that PAEE has significant antifungal activity and could be used to treat *T. mentagrophytes* infections in rabbits.

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INTRODUCTION

Dermatophytosis is a superficial mycotic fungal infection caused by various species of dermatophytes that affect the superficial keratinized tissues of both humans and animals (Dobrowolska *et al*., 2006; Iqbal *et al*., 2012). Dermatophytosis is a zoonotic disease and thus has important implications in public health (Cafarchia *et al*., 2012). *Trichophyton mentagrophytes* is one common species of dermatophytes that is equally important for man and animals. *T. mentagrophytes* is frequently found in rabbits (Veraldi *et al*., 2012) and causes serious infections in rabbits as well as subsequent economic losses to rabbit farmers (Cafarchia *et al*., 2010). Treatment of dermatophytosis usually involves the use of various antifungal agents such as clotrimazole, terbinafine, and ketoconazole (Rao *et al*., 1999). However, drug resistance, toxicities, and drug-drug interactions are the limiting factors. In traditional Chinese medicine, different parts of medicinal plants play different essential roles in ethnoveterinary medicine (Hoareau and Da silva, 1999) because of their effectiveness in treating the assorted ailments (Chan *et al*., 2008). About 40% of the total medicinal consumption in China is attributed to traditional medicines (Hoareau and Da silva, 1999). Antimicrobial, antifungal, and antioxidant properties of medicinal plant extracts have widely been reported (Kusuma *et al*., 2010). To discover new classes of antifungal agents that inhibit multi-drug resistance mechanisms, various research groups all over the world have conducted studies on medicinal plants (Abad *et al*., 2007). *Phellodendron amurense* is a Chinese medicinal plant that comes from a deciduous tree species widely grown in China. *Phellodendron amurense* is commonly used to treat various ailments including gastroenteritis, abdominal pain, diarrhoea, abscess, and other inflammations or swellings

(Cao *et al.*, 2004). However, the *in vivo* and *in vitro* inhibitory effect of the *Phellodendron amurense* ethanol extract (PAEE) against *T. mentagrophytes* has not yet been reported. Thus, this study aimed at exploring the *in vivo* and *in vitro* antifungal activity of the ethanol extract of the dried bark of *Phellodendron amurense* against *T. mentagrophytes* in rabbits.

MATERIALS AND METHODS

Extraction of PAEE: Four kilograms of dried *Phellodendron amurense* were finely ground using an electric blender and was extracted under reflux for 2 h with 75% ethanol at 80°C three times. After filtration and centrifugation at 1700×*g* for 30 min, the combined solution was concentrated under reduced pressure with a rotary evaporator at 65°C until a residual volume of 2000 mL of PAEE was reached (2 g/mL). PAEE was filtered through a 0.45-μm membrane filter and stored at 4°C for further experimentation.

Total alkaloid analysis of PAEE: We determined the levels of total alkaloids in PAEE as described by Xu *et al.* (2010). The level of total alkaloids in PAEE was calculated according to the standard curve established against the different concentrations of the standard solution. The experiment was carried out in triplicate.

Fungal organism: The eumycete isolated from dermopathic rabbits was obtained from Shaoxing District, and the Institute of Internal Medicine. Chinese Academy of Medical Sciences confirmed the presence of the *T. mentagrophytes* strain.

***In-vitro* antifungal assay:** The eumycete was grown on tryptic soya agar plates at 28°C for 4 days. The cultured material was collected by scraping the agar surface with a sterilized loop, and the material was shifted to a glass tube containing normal saline solution. The suspension was vortexed for 60s, and heavy particles were allowed to settle for 3-5 min. The densities of the suspension were adjusted spectrophotometrically to give primary inoculum of a final concentration of 1.0×10^6 CFU/mL in the normal saline solution.

Determination of the minimum inhibitory concentration (MIC): The minimum inhibitory concentration (MIC) is defined as the lowest concentration of a compound required to inhibit visual growth. Slight modification to the agar-diffusion method (Alagarsamy *et al.*, 2013) was used to assess the MIC. In brief, the serial volumes of PAEE (0.0, 0.5, 1, 1.5, 2, 2.5, or 3 mL) were gently mixed with 100 mL of tryptic soya agar while the serial volumes of clotrimazole (0.0, 0.5, 1, 1.5, 2, 2.5, or 3 mg) were dissolved in 0.2 mL of dimethyl sulphoxide. The clotrimazole served as the positive control. These mixtures were then poured into various sterile petri dishes and allowed to solidify by incubation at 45°C for 15 min. The final concentrations of *Phellodendron amurense* or clotrimazole were 0.00, 0.01, 0.02, 0.03, 0.04, 0.05, and 0.06 g/mL or 0.00, 0.005, 0.01, 0.015, 0.02, 0.025, and 0.03 mg/mL. Following this, 1.0×10^6 CFU/mL eumycete suspension was inoculated to petri dishes and incubated with 28°C with 60% humidity for 72 h. The MIC was taken as the lowest concentration of PAEE or clotrimazole required to inhibit growth of the fungus. Each experiment was carried out in duplicate.

Growth curve by dry weight determination: The time and concentration effect of PAEE and clotrimazole on *T. mentagrophytes* was determined through the method described by Alio *et al.* (2005). The eumycete culture (30000 cells/mL) diluted in 200 mL of tryptone soya broth was added in various conical flasks containing the known concentrations of PAEE (0.4, 0.8, 1.6, 2.0 g/mL) or clotrimazole (4.0 μg/mL) and then incubated at 37°C under continuous shaking at 170 cycles/min for 72 h. The data were recorded at 12, 24, 36, 48, 60 and 72 h.

Ultra-structural analysis by transmission electron microscopy (TEM): Transmission electron microscopy was used to observe the effect of the extract on the cellular ultra-structure as previously described (Basma *et al.*, 2011) with little modification. Fifty milliliters of *T. mentagrophytes* cells (3×10^5 CFU/mL) were treated with either 20 μL of distilled water or PAEE (0.8mg/mL) or clotrimazole (0.4μg/mL) for 16 h. Sections were observed with a JEM-1230 transmission electron microscope (JEOL Ltd., Japan).

Experimental animals: Thirty New Zealand white male rabbits aged 31 days, weighing 400-450 g, were purchased from the experimental animal centre at Zhejiang University, China. The Bioethics Committee of the Zhejiang Academy of Agricultural Sciences approved this experiment. The rabbits were divided into 5 groups consisting of 6 rabbits each. Three groups treated with PAEE were classified as PA group 1, PA group 2, and PA group 3. Two additional groups included the group not treated with PAEE i.e. the negative control (NC) group treated with distilled water or the positive control group (PC) treated with clotrimazole.

***In-vivo* antifungal assay:** Dermatophytosis was induced in rabbits as described previously (Mikaeili *et al.*, 2011). Briefly, a 6 × 4 cm area of skin was clipped from the middle of the backs of the test rabbits. A 1-mL suspension (1.0×10^6 cells) of *T. mentagrophytes* was applied to the marked area using a sterile pipette tip, and the area was rubbed thoroughly for 3 consecutive days. Different concentrations of PAEE were applied to PA group 1, 2, and 3 at 10%, 50%, and 100%, respectively on day 4 and again each following day for upto 7 days. Clotrimazole or distilled water was applied topically on day 4 and again each following day for upto 7 days in the PC group and NC group (Table 1). The lesions were evaluated continuously from the post-infection day to the tenth day. Clinical evaluation was according to the methodology previously described (Ghannoum *et al.*, 2008). The scores data from each treatment group were compared. Efficacy was calculated according to the formula described by Mikaeili *et al.* (2011) as follows:

Percent efficacy = 100-(T × 100/C) where,
T = total score of treatment group and C = total score of untreated control.

Table 1: Experimental animals challenged and received various treatments

Group	No. of animals	Challenge (day)	Treatment		
			Application	Day(s)	Quantity (per ml)
PA1	6	1, 2, 3	PAEE (10%)	4,5,6,7	0.2 g
PA2	6	1, 2, 3	PAEE (50%)	4,5,6,7	1.0 g
PA3	6	1, 2, 3	PAEE (100)	4,5,6,7	2.0 g
PC (Positive control)	6	1, 2, 3	Clotrimazole	4,5,6,7	0.01 g
NC (Negative control)	6	1, 2, 3	Distilled water	4,5,6,7	1 ml

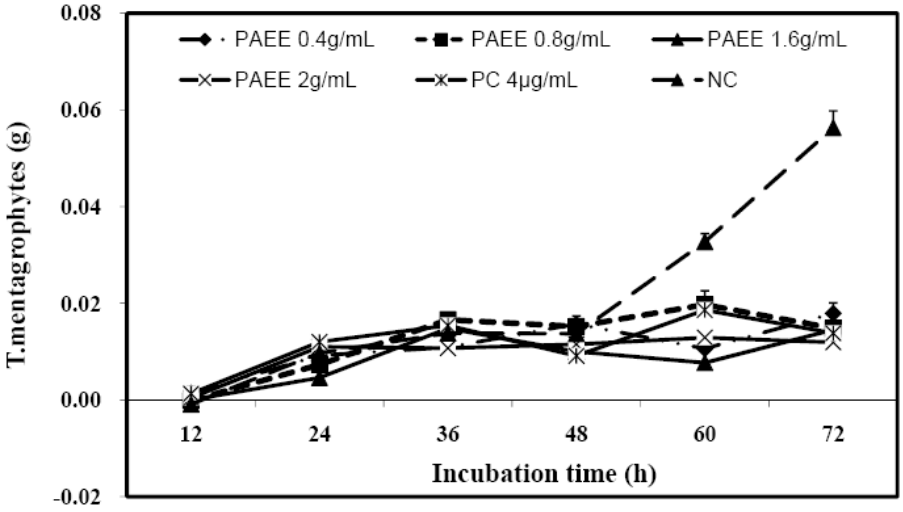

Fig. 1: The effect of PAEE on the growth of *Trichophyton mentagrophytes*. *Trichophyton mentagrophytes* were treated with PAEE (0.4, 0.8, 1.6, 2.0 g/mL) or clotrimazole (positive control, 4.0 µg/mL) or distilled water (negative control) subsequent for 72 h.

Table 2: Percent efficacy of all the treatment groups at the end of the experiment

Group	Percent efficacy
10% PAEE	81.9
50% PAEE	87.5
100% PAEE	86.1
Clotrimazole	83.3

Statistical analyses: Comparison of mean values for the *in vivo* antifungal assessment was carried out using one-way analysis of variance and the Turkey HSD test. A $P<0.05$ was considered statistically significant. Data were expressed as mean±SD.

RESULTS

Total alkaloids and MIC of PAEE or clotrimazole and Growth curve: The levels of total alkaloids in PAEE was calculated according to the equation of the standard curve, $Y = 0.0022X + 0.3314$ ($R^2 = 0.9494$). We found the levels of total alkaloids in PAEE to be 7.58±0.46 mg/mL.

The MICs were determined after 72 h. The MIC for PAEE and clotrimazole were 0.03g/mL and 0.02 mg/mL, respectively. The growth inhibition of *T. mentagrophytes* according to different concentrations of PAEE and clotrimazole that started after 48 h is shown in Figure 1.

Ultrastructure analysis: In TEM observations, the picture of cell membrane and cell wall were clearly seen in normal *T. mentagrophytes* (Fig. 2 A). The cell membrane of *T. mentagrophytes* was disrupted by PAEE and clotrimazole (Fig. 2 B and C).

***In-vivo* antifungal assay:** On day 4, no significant recovery was evident in any group; however, on day 5, significant recovery of skin lesions was observed only in the PC group when compared with the NC group ($P<0.05$). The animals were further observed from day 6 to 10, and this revealed a significant recovery of skin lesions in all treatment groups (PA group 1, 2, 3, and the PC group) when compared with the NC group. The recovery of the lesions among the treatment groups on each day was also compared. No significant differences among all the treatment groups were recorded on days 4, 7, 8, 9, or 10. However, on days 5 and 6, a significant difference in PA group 3 and the PC group was recorded when compared with PA group 1 and PA group 2 ($P<0.05$) (Fig. 3). In the control group, the lesions were significantly high on day 5 compared with the first day of post negative treatment. The efficacy of PAEE in the treatment groups is shown in Table 2. Although there were no significant differences in the efficacy among all the treatment groups at the end of the experiment, the efficacy in PA group 2 (50% PAEE) was higher than that in the other groups.

DISCUSSION

Dermatophytes are pathogenic fungi that have the ability to invade a keratinized structure and infect the skin, hair, and nails of humans and animals. The genera *Trichophyton* is found in man and animals, and the specie *T. mentagrophytes* is zoonotic in nature. *T. mentagrophytes* is major cause of dermatophytosis in pets and rabbits. Drouot *et al.* (2009) isolated 38.1, 7.1, and 29.3% of the *T. mentagrophytes* complex from guinea pigs, dogs, and cats, respectively. *T. mentagrophytes* is the most common fungus identified in rabbits, and a considerable increase in human dermatophytosis,

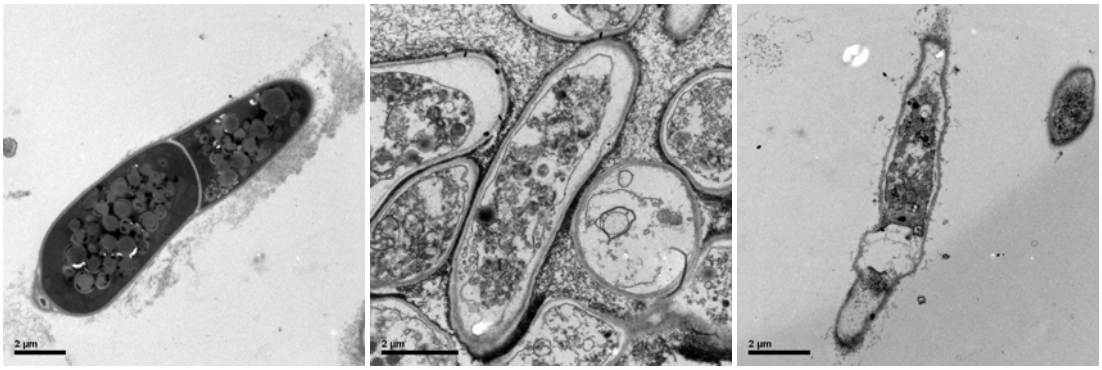

Fig. 2: Ultrastructure of *Trichophyton mentagrophytes* cell. *Trichophyton mentagrophytes* cells were treated with PAEE, and were observed by transmission electron microscopy. (A) Normal ultrastructure of *T. mentagrophytes* cell (negative control); (B) ultrastructure of *Trichophyton mentagrophytes* cell treated with PAEE; (C) ultrastructure of *Trichophyton mentagrophytes* cell treated with clotrimazole as the positive control. The cell membrane of *Trichophyton mentagrophytes* was seriously destroyed by PAEE or clotrimazole (arrows indicate destroyed cell membrane).

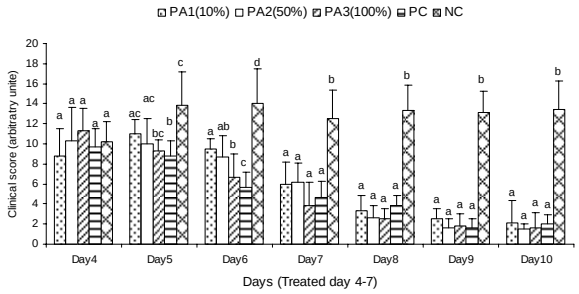

Fig. 3: Effect of ethanol extract of *Phellodendron amurense* (PAEE-10%, 50% or 100%), Clotrimazole (Positive control) or distilled water (Negative control).Significant differences are mentioned with different alphabets at the level of P<0.05 and P<0.01.

transmitted by rabbit, has been observed (Gallo *et al.*, 2005). Due to the zoonotic transmission of *T. mentagrophytes*, efforts toward effective treatment are necessary, and various parts of traditional medicinal plants were reported to be effective against dermatophytosis (Zhang *et al*., 2006).

Phellodendron amurense has been used in traditional Chinese medicine to treat inflammation and swelling (Cao *et al.*, 2004). Sanhuang liniment, a Chinese herbal medicine, contains *Phellodendron amurense* for use as an effective antifungal treatment. This medicine is used to treat skin pruritus, furuncle carbuncle, acute eczema, impetigo, and skin infections (Wu *et al.*, 2009). Tang *et al.* (2008) studied the inhibitory effects of the crude ethanol extract of the *Phellodendron* fruits and its 4 different polar fractions against 11 phytopathogenic fungi such as *Rhizoctonia cerealis* and *Rhizoctonia solani*. They found that both ethylacetate and n-butanol fractions showed stronger inhibitory effects on the fungi (Tang *et al.*, 2008). To our knowledge, the antifungal effect of PAEE against *T. mentagrophytes* in rabbits has not been studied thus far. The present study revealed the antifungal effect of PAEE against *T. mentagrophytes* in rabbits *in vivo* and *in vitro*. In addition, the MIC of PAEE was 0.03 g/mL (1.5%), which is much lower than that of the aqueous extract (10%) previously reported (Qiu and Yu, 2007). This suggests that there are more active antifungal ingredients in PAEE than in the aqueous extract. Alum and gleditsia were reported to be the most effective antifungal agents against *T. mentagrophytes*, and their

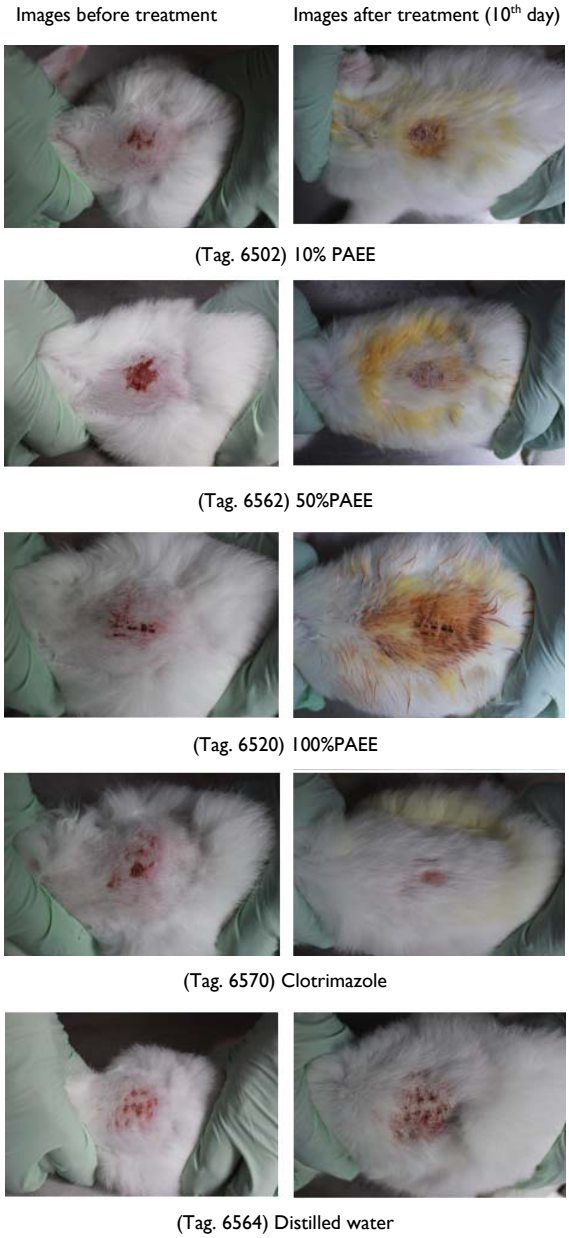

Fig. 4: Efficacy of PAEE or clotrimazole or distilled water against *T. mentagrophytes* in rabbits. The images were taken on Day 10.

MIC was found to be 1.25% (Qiu and Yu, 2007), which was almost the same concentration as in our study (1.5%).

Several studies have documented the antifungal activities of various alkaloids extracted from medicinal plants (Emile *et al.*, 2007; Orhana *et al.*, 2007; Zhou and Zhang, 2008). Berberine is the main active ingredient in traditional Chinese medicine, which is often used as a quantitative index in the preparation of *Cortex Phellodendri*. In the present experiment, the total alkaloid level in PAEE was 7.58±0.46 mg/mL. These findings are not in agreement with the results of a previous study by Fan and Zhang (2010). Our results suggest that a strong antifungal activity is possible with a low level of alkaloids. Transmission electron microscopy images showed that the cell membrane of *T. mentagrophytes* was destroyed by PAEE (0.8 mg/mL) and clotrimazole (0.4 µg/mL).

In the *in vivo* study, it was observed that the lesions in the PAEE treatment groups started to recover from day 6 until day 10. No significant differences between 50% and 100% PAEE treatment groups were found; therefore, PAEE concentration was most likely sufficient in this range. Treatment with clotrimazole also subsided skin lesions, but there was no significant difference when compared with PAEE. Clotrimazole is well-documented antifungal agent, and the performance of PAEE in this study was in good agreement with the performance of clotrimazole; thus, PAEE is also suggested to have antifungal potential. The *in vivo* antifungal activity of all PAEE treatment groups compared with the control group showed significant differences ($P<0.05$ or $P<0.01$). In the control group, skin lesions were slightly improved on day 7 post treatment, but there was no significance difference when compared to other days. This might be explained by the influence of the rabbits' immune response against the *T. mentagrophytes* infection (Zrimsek *et al*., 2003).

Conclusion: PAEE was a significant antifungal agent both *in vitro* and *in vivo* against *T. mentagrophytes* and could be used as an alternative medicine for the treatment of dermatophytosis in rabbits.

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