

Original Article

Effect of pH on fungal growth: problems with using vinegar (5% acetic acid) in treating superficial fungal infections

酸鹼值對真菌生長的影响：用醋（5%醋酸）治療表層真菌感染所面對的問題

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Objectives: To investigate the effect of different pH on the *in vitro* growth of *Trichophyton rubrum* (*T. rubrum*) and to study the pH achievable by a test product at various depths in a porcine nail model. **Methods:** *T. rubrum* was grown in Sabouraud dextrose broth of various acidic pH to determine the fungicidal level. A test product with active ingredient of acetic acid was applied to the porcine nails for 60 and 120 times and the pH at various depths of penetration was measured by pH metre. **Results:** A pH of 3.0 or below is fungicidal to *T. rubrum* and the minimal pH achievable by 60 and 120 applications of the test product were 4.09 and 3.37 respectively. **Conclusions:** Use of vinegar (5% acetic acid) to treat tinea pedis is theoretically efficacious but it is difficult to achieve a fungicidal pH of 3.0 at the nail bed despite prolonged application.

目的：研究不同酸鹼值對活體外紅色毛癬菌生長的效果，並檢測所測試產品在豬蹄甲模型的不同深度可達致的酸鹼值。**方法：**在不同酸鹼值的薩氏葡萄糖瓊脂培養基內培養紅色毛癬菌，以確定殺真菌的酸鹼水平。另將一種內含醋酸活性成份的測試產物分別塗於豬蹄甲各60和120次，然後用酸鹼度計探測不同深度滲透所達致的酸鹼值。**結果：**紅色毛癬菌的殺真菌酸鹼值水平為3.0或以下，而該測試產品在60和120次塗抹後可達致的最低酸鹼值分別為4.09和3.37。**結論：**使用醋（5%醋酸）來治療足癬，在理論上是可行的；但即使長時間應用，它亦難以在甲床達致3.0的殺菌酸鹼值水平。

Keywords: Acetic acid, pH, superficial fungal infections, *T. rubrum*, vinegar

關鍵詞：醋酸、酸鹼值、表層真菌感染、紅色毛癬菌、醋

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Introduction

Superficial fungal infection is a common skin problem.¹ In a local study, tinea pedis and tinea unguium account for 20.4% and 16.6% of skin clinic attendants respectively.² The study also found *Trichophyton rubrum* (*T. rubrum*) to be the commonest dermatophyte causing tinea pedis and onychomycosis which was in agreement with a previous Hong Kong study.^{2,3} The psychosocial impact of this disease among those severely infected is significant.⁴ Cure of the disease is not difficult as there are a number of effective

medicaments.⁵ Topical antifungals are usually sufficient for treating superficial skin infections such as tinea pedis. However, the treatment of nail infection often requires oral medications due to the poor penetration of the topical agents.⁶ With the improvement in the transungual delivery technique, on the other hand, newer topical antifungals can achieve a cure rate above 50%.^{7,8}

The use of herbal extracts in the treatment of superficial fungal infection is not new.⁹ Among the folklore remedies, vinegar (5% acetic acid) has been one of the agents used to treat tinea pedis and tinea unguium. The mechanism of action is not clear though it has been suggested that the acidity (low pH) of the vinegar may have a fungistatic or even fungicidal activity.¹⁰ Commercial products using this strategy are mushrooming in the internet claiming a high efficacy of this method. Yet few scientific studies have been performed to substantiate these claims.

In this study, we investigated the effect of different pH on the growth of *T. rubrum* *in vitro*. In addition, we studied the pH achieved at various depths in a porcine nail model by an acetic acid-containing product.

Methods

Culture of *Trichophyton rubrum* in various pH media

I. Strain

Type strain of *T. rubrum* ATCC 28191 was cultured on Sabouraud dextrose agar plates (Oxoid Ltd, Cambridge, Cambridgeshire, United Kingdom) at 25°C for 14 days. Conidia were harvested by gently swabbing the culture growth with sterile swabs and then re-suspending in sterile 0.9% saline to a final concentration of 5×10^3 conidia/ml. The conidia suspension was freshly prepared for each experiment.

II. Establishment of different pH solutions

Sabouraud dextrose broth (Oxoid Ltd, Cambridge, Cambridgeshire, United Kingdom)

was used for establishing different pH solutions. The broth was adjusted to various pH levels, namely 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 with 37% HCL or 1M NaOH. The final pH was determined by a pH meter (Pinnacle M530P, SI Analytics GmbH, Germany). Aliquots of 25 ml solutions were then dispensed into sterile conical falcon tubes. The tubes were maintained at 25°C before use.

III. T. rubrum cultures at different pH values

Each pH solution was inoculated with 0.2 ml of the conidia suspension and incubated at 25°C for 14 days. Dry weight was determined by harvesting the mycelia on day 15 through Whatman filter paper (Whatman, GE). The retentate was then dried and weighed.¹¹ The pH of the broths was recorded at the end of incubation. Three replicates of each treatment were performed.

pH at various depths of porcine nail after application of the test product

I. Preparation of porcine hoof nail plates and drug application

Nail plates were removed from porcine hooves with the underlying cartilage and connective tissue trimmed. They were then disinfected in 70% (v/v) ethanol for 24 hours followed by rehydration in normal saline for the same period. Sections of 2x2 cm² were sampled from the uniform part of the nail plates after careful inspection to avoid pores or cracks. The nail sections were then placed on sponges soaked in normal saline and kept in a ventilation chamber. The temperature and relative humidity within the chamber were maintained at 25±3°C and 78±5% respectively (Figure 1). After the nails returned to its original hydration state, the test product, (Excilor®: Ethylactate, aqua (water) (7%), acetic acid, penetration enhancer, film-forming agent, preservatives), were applied evenly over the surface of each nail plate (roughly equivalent to 2 µl/cm²). Each application was separated by at least 30 minutes allowing the nail surface to dry. Two treatment groups treated with 60 applications

(five times per day for 12 days) and 120 applications (five times per day for 24 days) were studied. The control group did not receive any topical application. Each group consisted of two nail samples.

II. Thickness and pH measurement

The nail plate was patted dry with tissue paper and mounted onto a drilling platform. The thickness and pH of the nail plate were measured with a micrometre screw gauge and pH metre (PH905 Courage Khazaka Electronic GmbH, Germany) correspondingly. A thin layer (approximately 0.3 mm) from the top of the nail plate was drilled off each time. After

measuring its depth with the micrometre screw gauge, the pH value at that particular depth was also documented. The procedure was stopped at a depth of 1.5 mm which was more than the usual 1.0 mm thickness of human nail (Figure 2).

Statistical analysis

Continuous variables were analysed by the Student's *t* test. A *p*-value of less than 0.05 was taken as significant. A non-linear regression model was used to calculate the pH at designed depth of penetration for the treated and control nails (SPSS V15 Chicago, Illinois, United States).

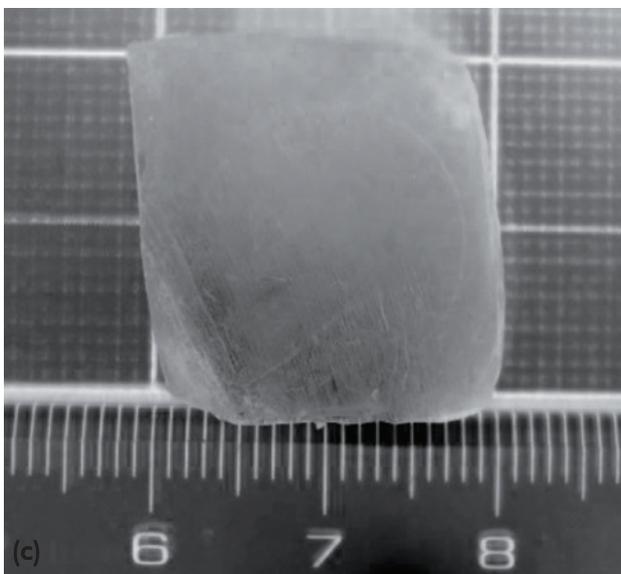
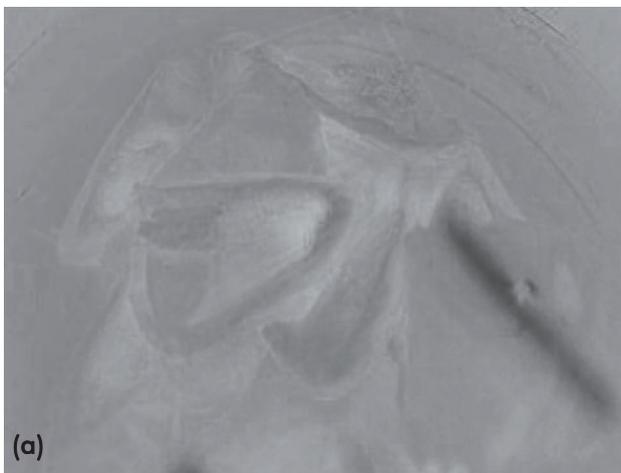


Figure 1. (a) Nails removed from pig's hoof. (b) Nail plate soaked in 70% alcohol for disinfection. (c) A 2x2 cm² section was sampled. (d) Nail plates kept in the ventilating chamber.

Results

Growth of *Trichophyton rubrum* in various pH media

Sabouraud dextrose broths of different pH values were established and maintained for 14 days. No significant difference in pH was found between those with and without *T. rubrum* inoculation (Student's t test, $p < 0.05$).

At the end of the incubation period, no growth was detected for those broths with pH of ≤ 3.0 . In order to ensure that the conidia in those broths were dead, they were sub-cultured in Sabouraud dextrose agar with pH 5.5 for 14 days. These

again showed no growth. For broths with $\text{pH} \geq 3.5$, fungal growth was observable. The effect of different pH on the amount of growth (dry weight) was shown in Table 1; namely, the mean dry weight of the mycelia harvested from the broths with pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 were 0.21, 0.22, 0.75, 1.11, 0.77, 1.28 and 1.00 mg respectively. Their dry weight ratios are shown in Figure 3.

Adjusted pHs at various depths of treatment and control groups

The adjusted pHs at various depths of the treatment groups (60x and 120x) and the control are shown in Table 2 and Figure 4.

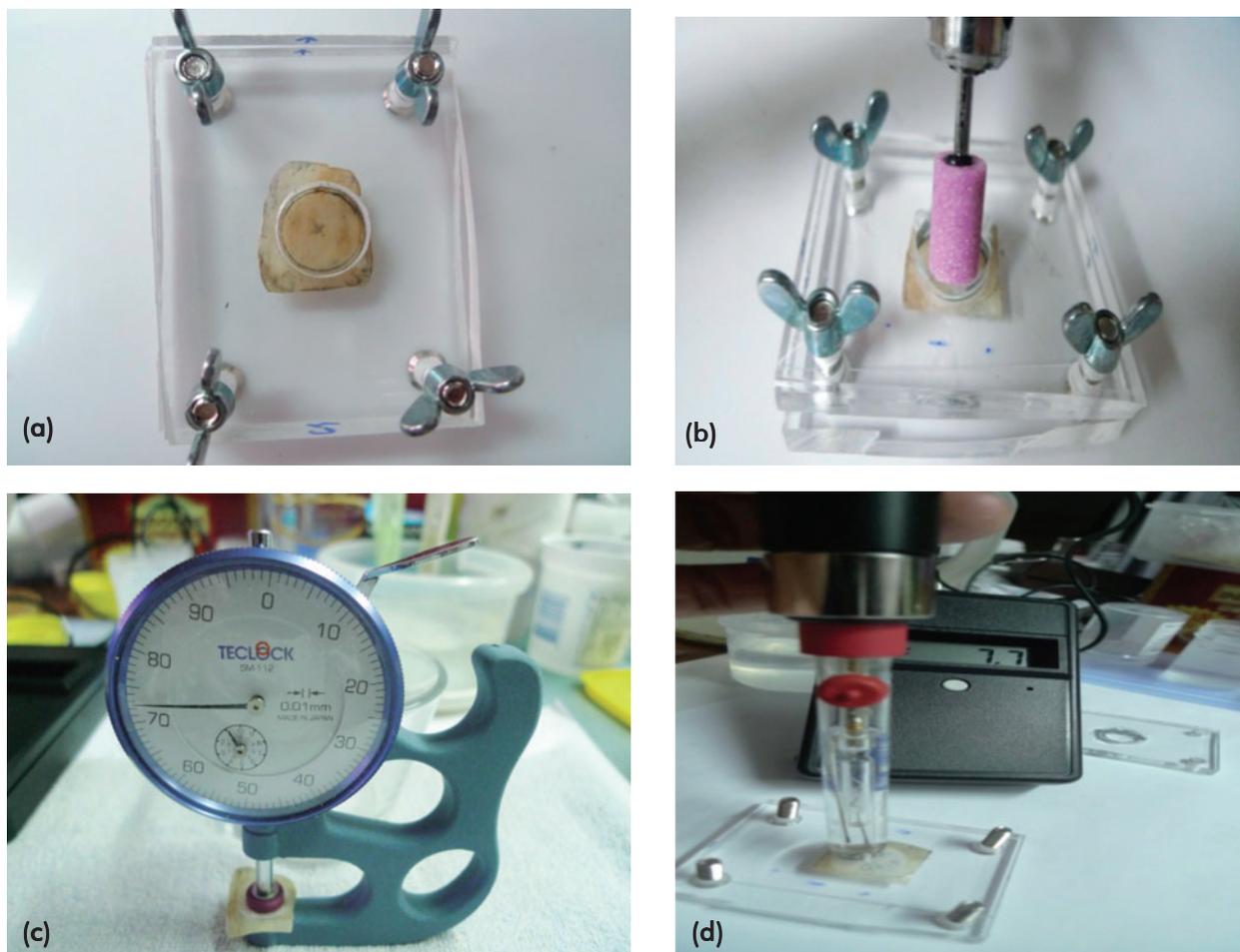


Figure 2. (a) Porcine nail plate mounted on the drilling platform. (b) Drill head inserted through the hole for drilling. (c) Micrometre screw gauge measuring the depth after drilling. (d) pH meter measuring the pH after drilling.

For the nails treated with 60 applications, the adjusted surface pH was found to be 4.37. This dropped slowly to a minimum of 4.09 at the depth of 0.25 mm. Beyond this depth, the pH quickly rose back to 6.39 at 1.0 mm. At 1.5 mm, it

reached 7.14. A similar trend was observed for the nails treated with 120 applications. Their adjusted surface pH was 4.12 which dropped gradually to a minimum of 3.37 at 0.5 mm and steadily rose back to 3.84 at a depth of 1.0 mm.

Table 1. Effect of different pHs on the growth of *T. rubrum* ATCC 28191 measured by dry weight

pH	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5
Dry weight, mg (Mean±SE)	0.00	0.00	0.00	0.21±	0.22±	0.75±	1.11±	0.77±	1.28±	1.00±
				0.08	0.06	0.35	0.52	0.28	1.02	0.53

Detection limit of the weight: 0.01 mg

Table 2. Adjusted pH at designed depth of penetration for treatment and control groups

Depth of penetration (mm)	Adjusted pH at 60 applications ^α	Adjusted pH at 120 applications ^β	Control ^γ
0.00	4.37	4.12	7.69
0.25	4.09	3.51	7.50
0.50	4.56	3.37	7.30
0.75	5.44	3.54	7.17
1.00	6.39	3.84	7.14
1.25	7.07	4.09	7.27
1.50	7.14	4.13	7.59

^α: Cubic model, R square 0.969, $p < 0.001$; ^β: Cubic model, R square 0.59, $p = 0.003$; ^γ: Cubic model, R square 0.968, $p = 0.002$

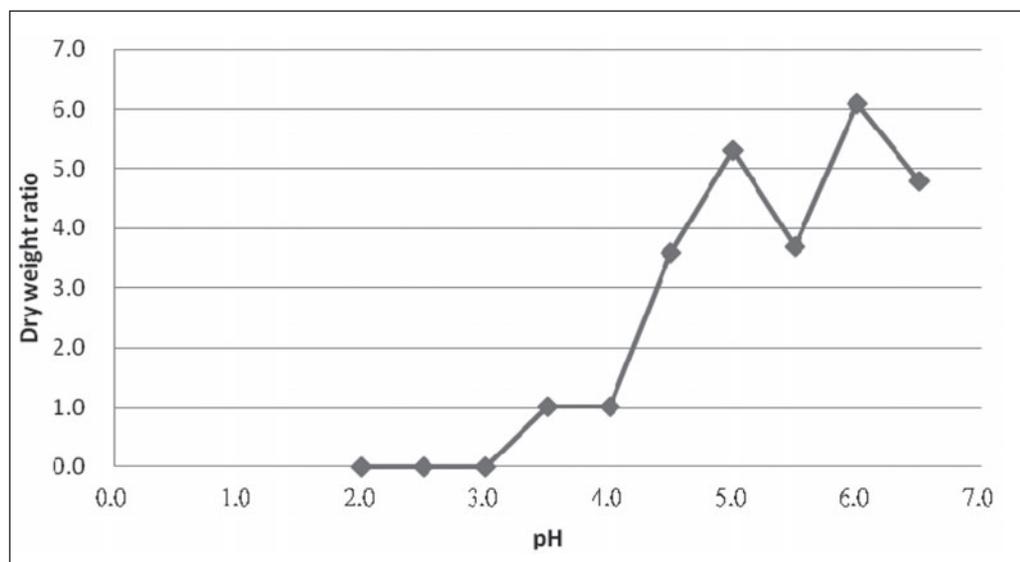


Figure 3. Dry weight ratio at various pH values.

At 1.5 mm, it reached a pH of 4.13. For the control nails, the pHs at different depths varied between 7.14 and 7.69 (Figure 4).

Discussion

Dermatophytes can infect human skin and nail causing tinea pedis and onychomycosis. At present, there are a number of effective antifungal drugs such as the polyenes, azoles and allylamines. Vinegar is used in traditional Chinese medicine (5% acetic acid) to treat these infections. Patients with tinea pedis are required to immerse their feet into warm-to-hot diluted acetic acid solution for 30 minutes.

Our study showed that *T. rubrum*, the main causative organism in causing these infections in Hong Kong, can survive an acidic environment of $\text{pH} \geq 3.5$. The fungal inhibitory effect seems to lessen with increasing pH as shown by the increasing amount of fungal growth at the higher pH values.

Vinegar or 5% acetic acid, at different dilutions, can achieve a pH of between 2.5 and 3.3. Theoretically it is effective in eradicating the dermatophytes in tinea pedis and even tinea unguium if nail bed penetration is adequate. However, there are problems with this.

Firstly, prolonged immersion of the epidermis in a low acidic environment would probably affect its barrier function. It is well known that the stratum corneum is a protective layer against external hazards, may it be chemical or microbial.¹² A normal stratum corneum requires the continual synthesis of the lipid layer which involves several pH-dependent enzymes.¹³ Among these are β -glucocerebrosidase, acid sphingomyelinase, acid lipases, phosphatases and phospholipases.¹⁴ The optimal pH condition for β -glucocerebrosidase in the synthesis of the important ceramides is 5.6. It is also known that the acidity in the extracellular space of the stratum corneum may affect the intracellular enzymatic activities.^{15,16} Hence exposing the stratum corneum to a pH of 3.5 or below for a prolonged period of time would

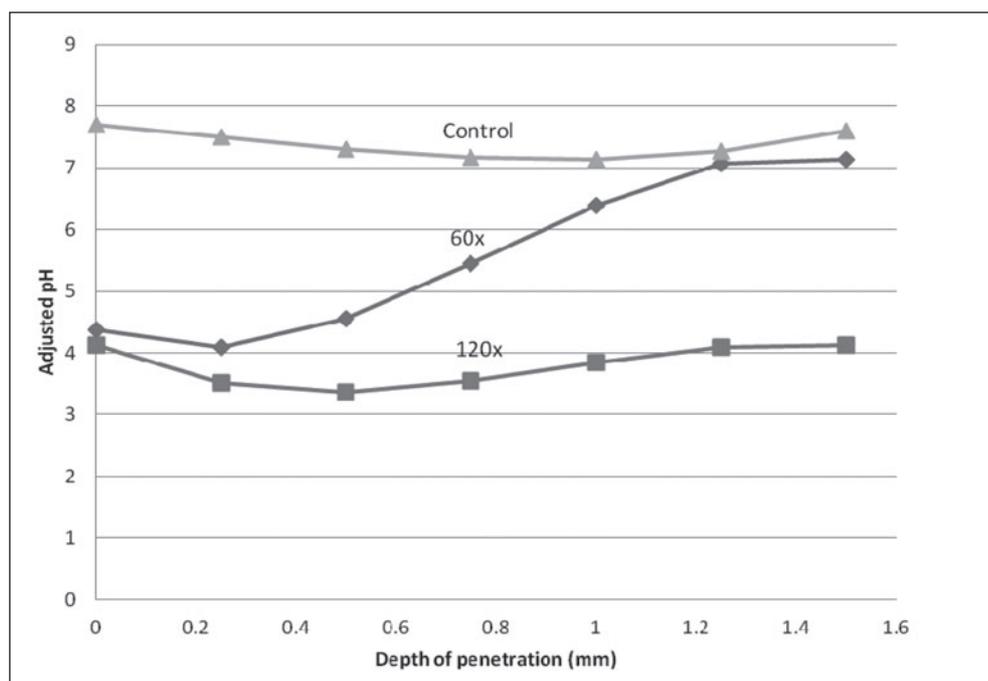


Figure 4. Adjusted pH at various depths of penetration of treatment (60x and 120x) and control groups.

probably affect its lipid content and barrier function. Besides, this warm-to-hot foot soaking method is not recommended for those with cardiovascular disease as the prolonged vasodilation induced may put extra stress on the heart.

Secondly, in our porcine nail model, the minimum pH achieved after 60 applications and 120 applications of the test product was 4.09 and 3.37 respectively. Both were above the fungicidal pH of 3.0. Besides, these pH values were attained at the superficial parts of the nail only. Hence, it is unlikely that fungal infection would be eradicated as the nail bed is more than 1.00 mm thick. Nevertheless, our study did show that lower pH values were attained with increased applications of this test product. Thus, it is possible that with a sufficient number of applications, a fungicidal pH could eventually be achieved at the nail bed.

Limitation of the study

The pH developed in our culture broths was achieved using hydrochloric acid and sodium hydroxide. These were used because a wide range of pH values (2.0 to 6.5) could be achieved with just a small quantity of the reagents. This has an advantage of preserving the concentration of other constituents in the medium. On the other hand, this study assumes that pH is the only factor that affects the growth of the fungus while neglecting the effect of the acidifying agent itself. Further studies are required using vinegar (5% acetic acid) as the acidifying agent to compare the fungicidal pH with that obtained in this study. In addition, the pH interval between the broths was wide i.e. 0.5, therefore the actual fungicidal pH cannot be ascertained accurately though it might lie between pH of 3.0 and 3.5.

The standard errors when measuring the biomass of *T. rubrum* by dry weighing of the mycelia appeared to be larger than desired. This could be due to the low concentration of the conidia

suspension inoculated. Future studies using a higher concentration of the conidia suspension may reduce this error.

The porcine nails model was employed in this study as it has been well described in the literature,¹⁷ yet we realise that there is a significant difference in composition between the human nail and porcine nail. One thing of note is that the number of sulphide linkages in porcine nail is less than that of human nail, enabling easier penetration.¹⁸ Furthermore, we applied the test product at a much higher frequency than that recommended by the manufacturer. Nonetheless, we adopted this method for the interest of time and in maintaining the integrity of the porcine nails. Further studies employing twice daily application may eliminate any bias due to this 'intensive' method of application.

Conclusion

Vinegar (5% acetic acid) is theoretically efficacious in treating tinea pedis, but may jeopardise the epidermal barrier function. It is difficult to achieve a fungicidal pH of 3.0 in the nail bed with the test product despite prolonged application.

Conflict of interest

This study is sponsored by Galderma Hong Kong Ltd.

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