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In vitro antifungal efficacy of ciclopiroxolamine alone and associated with zinc pyrithione compared to ketoconazole against *Malassezia globosa* and *Malassezia restricta* reference strains

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Abstract

The aim of this study was to determine the *in vitro* fungicidal and growth inhibitory activity of ciclopiroxolamine alone (1% and 1.5%) or in association with 1% zinc pyrithione compared to 2% ketoconazole, against *Malassezia* species particularly involved in the pathogenesis of seborrheic dermatitis. Experiments were performed on *Malassezia globosa* IP 2387.96 and *M. restricta* IP 2392.96 strains. Growth inhibitory activity of the active compounds in solution was evaluated by measuring minimal inhibitory concentrations using a broth micro-method and their fungicidal activity by a filtration method after contact times between solutions and yeasts ranging from 3–5 to 30 min. Concerning the determination of minimal inhibitory concentration of ciclopiroxolamine/zinc pyrithione, it revealed the marked synergistic inhibitory effect of the association, leading to a higher efficacy compared to ketoconazole. As to the fungicidal activity of ciclopiroxolamine, it significantly increased with the contact time. After 15–30 min of contact between 1.5% ciclopiroxolamine and *Malassezia* strains, a 2-log reduction of *Malassezia* counts was observed. The 1.5% ciclopiroxolamine/1% zinc pyrithione association was characterized by a steady fungicidal efficacy whereas the 2% ketoconazole solution did not express any fungicidal effect. In conclusion, this study demonstrates the *in vitro* inhibitory and fungicidal efficacy of the ciclopiroxolamine/zinc pyrithione association against *Malassezia* species and underscores its potential interest in the treatment of seborrheic dermatitis.

Key words: ciclopiroxolamine, fungicidal agent, growth inhibition, *Malassezia*, zinc pyrithione.

Abbrevations: SD – Seborrheic dermatitis; CPO – Ciclopirox olamine; ZP – Zinc pyrithione; MIC – Minimal Inhibitory Concentration

Introduction

Seborrheic dermatitis (SD) is a common, chronic, superficial inflammatory skin disorder covering areas of the scalp, face and trunk [1] and affecting 1%–3% of the population. Dandruff could be related to this disease via a common etiology [2]. Recent studies have demonstrated that SD pathogenesis involves host factors [3] and lipophilic yeasts of the genus *Malassezia* [4–7] in overabundance, among which *M. restricta* and *M. globosa* appear to be the most frequently isolated [2, 8–10].

The standard treatment of SD/dandruff consists of antimycotic shampoos to control *Malassezia* development [1, 11] containing azoles and especially ketoconazole [12, 13], hydroxypiridones [14–17] or various agents such as zinc pyrithione

[18, 19], tar and selenium disulphide. However, though these treatments are able to achieve clinical improvement, their discontinuation is often followed by *Malassezia* yeasts recolonization, which is responsible for the main part of symptoms recurrence. With the aim at improving current therapeutic strategies of SD, recent clinical studies have evaluated different combinations of potent antimycotic agents [20–22]. But prior to clinical trials, the interest of such associations in reducing recolonization must be explored in *in vitro* studies in order to define their antifungal activity on *Malassezia* cells not only in terms of growth inhibition but also of fungicidal effect, due to the specific mode of use of shampoo-based SD treatments, which consists in applying high doses of active compound during a short contact time.

Therefore, the present study was performed to evaluate the growth inhibitory activity and the fungicidal effect of the ciclopiroxolamine/zinc pyrithione association (CPO/ZP) against *Malassezia globosa* and *M. restricta* reference strains, compared to CPO and ZP alone and to ketoconazole, the referent molecule used in the treatment of SD.

Materials and methods

Chemicals and test solutions

The hydrophilic solutions of 1% (10 g/L) and 1.5% (15 g/L) CPO (w/v), 1% (10 g/L) ZP (w/v) and 1.5% (15 g/L) CPO/1% (10 g/L) ZP combination (w/v) have been provided by the Institut de Recherche Pierre Fabre (France). A solution of 2% (20 g/L) ketoconazole (Sigma) prepared in 10% DMSO was used as control. Hydrophilic neutral solutions (without active compounds) used were tested in the same conditions.

Malassezia strains

The strains were obtained from Pasteur Institute Fungi Collection (Paris, France) and corresponded to the major species implicated in dandruff: *Malassezia globosa* IP 2387.96 and *Malassezia restricta* IP 2392.96. Strains were incubated during 5 days at 30 °C on modified Dixon agar under aerobic conditions. The minimal inhibitory concentrations (MICs) were determined in modified Dixon broth. For fungicidal tests, numerations were carried out after incubation on Dixon agar during 5 days at 30 °C under aerobic conditions. The suspensions were prepared in sterile distilled water using glass beds for dispersion just before use, at a concentration of 10^7 yeast cells/mL as controlled by microscopic counts.

Methods

- The growth inhibitory activity was determined by measuring the MICs using a broth micromethod. Briefly, 100 μl of modified Dixon broth were placed in each well of two 96-well microtiter plates and 100 μl of the test solutions were added to the first column of the first plate. In order to obtain an extended range of tested concentrations, two-fold dilutions were carried out from one column to the next, up to fill all the columns of the 2 microplates, except columns 11 and 12 that were respectively used as control of the medium sterility (without test solution and without spores) and growth (without test solution, with inoculum). The microplates were inoculated using a multipoint inoculator (Denley) to obtain a final concentration of 10^5 cells/mL, and incubated during 5 days at 30 °C. After control of the growth in each well of the column 12, MIC was determined as the first concentration corresponding to no visible growth (mg/L). Assays were performed in duplicate.
- The fungicidal activity of the hydrophilic solutions was determined according to the ''filtration'' method adapted from the European Standard [23]. Briefly, 9 mL of each solution was mixed with 1 mL of *Malassezia* suspension. After 3–5, 15 and 30 min of contact at 32 °C, the reaction was stopped by filtering 1 mL of the mixture and the 10-fold dilutions with sterile distilled water (10^{-1}–10^{-3}) onto a 0.45 μm membrane. After rinsing, the membrane was put on modified Dixon agar and incubated as described above. The innocuousness of the excipients and the validity of the neutralization by filtration were verified according to the Standard (preliminary tests). The initial inoculum was determined by filtration of 10^{-4} and 10^{-5} dilutions of the initial suspension. Assays were performed twice when the tested solutions had no fungicidal effect, and four times for solutions expressing

potent fungicidal effects. Results were expressed as the percentage or the base-10 logarithm of the reduction in residual viable counts compared to the initial inoculum. Statistical analysis was performed using a paired test for the evaluation of contact time effect and an unpaired test for the comparison between tested solutions.

Results

Determination of the growth inhibitory efficacy

Table 1 indicates the values of MIC (mg/L) for CPO, ZP, ketoconazole and CPO/ZP association. Compared to ketoconazole, ZP expressed a similar high inhibitory activity but the most significant result was observed for CPO/ZP association, which proved to be more efficient than ketoconazole. Indeed, the combination of the two inhibitory compounds showed a highly synergistic effect with a 10–100-fold decrease in the efficient MIC compared to CPO and ZP alone and to ketoconazole. In the assay conditions, hydrophilic neutral solutions express no inhibitory effect.

Kinetics of fungicidal activity

The mean reduction percentage in residual viable counts for *M. globosa* and *M. restricta* was compared to the initial inoculum for the 1% CPO, 1.5% CPO and 1% ZP solutions (Figures 1 and 2). Hydrophilic neutral solutions did not show any detectable fungicidal activity. For both strains, the 1.5% CPO solution induced higher percentages of reduction in residual viable counts than the 1% solution for all contact times, including the short one. A significant time-dependent effect ($P < 0.05$) was observed for the 1.5% CPO solution, leading to a count reduction of 90% and 99% after respectively 15 and 30 min of contact for both strains tested. By contrast, the 1% ZP solution showed a poor fungicidal efficacy, as it did not reduce *M. globosa* counts and only slightly *M. Restricta* counts, even after 30 min of contact. The fungicidal activity of CPO and ZP solutions appeared to be slightly more efficient on the *M. restricta* strain than on the *M. globosa* strain but without significant difference between both strains.

The same experiments were performed with the 1.5% CPO/1% ZP association and 2% ketoconazole solution. The kinetics of mean reduction for the two solutions according to the contact time are presented on Figures 3 and 4, for *M. globosa* and *M. restricta* strains respectively. The CPO/ZP combination showed a potent fungicidal time-dependent efficacy, similar to that observed with the CPO solution alone, confirming the absence of an antagonist effect between the two compounds. By contrast, the 2% ketoconazole solution did not express significant fungicidal activity (log reduction ≤ 0.5) even after 30 min of contact. Hence, the difference of fungicidal efficacy between the 1.5% CPO/ZP and 2% ketoconazole solutions was statistically significant as early as 3–5 min of contact for *M. restricta* and 15 min of contact for *M. globosa* ($P < 0.05$), reaching a high significance ($P < 0.01$) after 30 min of contact for both strains.

Table 1. MICs (Minimal Inhibitory Concentrations) (mg/L) of ciclopiroxolamine, zinc pyrithione, ketoconazole and the association ciclopiroxolamine/zinc pyrithione against *Malassezia globosa* IP 2387.96 and *Malassezia restricta* IP 2392.96

	MICs (mg/L)	
	M. globosa IP 2387.96	*M. restricta* IP 2392.96
Ciclopirox olamine	7.3	7.3
Zinc pyrithione	0.3	0.15
Ketoconazole	0.5	0.5
Ciclopirox olamine/ Zinc pyrithione (1.5/1)	0.014/0.009	0.014/0.009

Discussion

Our results confirm the real fungicidal efficacy of hydroxypyridones [24, 25] like Ciclopirox olamine against *Malassezia* yeasts, underscore the highly potent growth inhibitory effect of zinc pirithione and demonstrate the higher fungicidal and fungistatic efficacy of its association with CPO compared to ketoconazole.

The mode of action of hydroxypyridones is very complex, targeting a variety of metabolic processes in the fungal cell [26], inhibiting the uptake of essential substrates and ions, thus leading to intracellular depletion [27] associated with topical anti-inflammatory properties [28]. The main

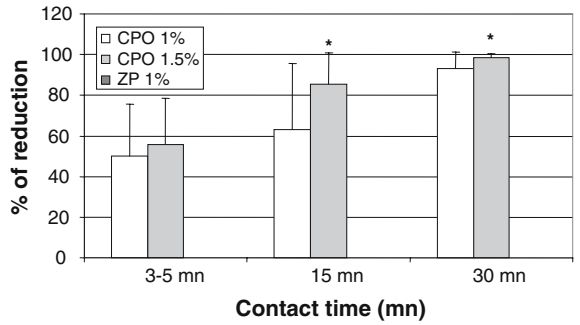

Figure 1. Evolution of the percentage reduction in viable counts (mean ± standard error) of *Malassezia globosa* IP 2387.96 according to the contact time (min) with the 1% ciclopiroxolamine (CPO), 1.5% CPO and 1% zinc pyrithione solutions (no detectable logarithmic reduction). $*P < 0.05$ in comparison with the 3–5 min contact time.

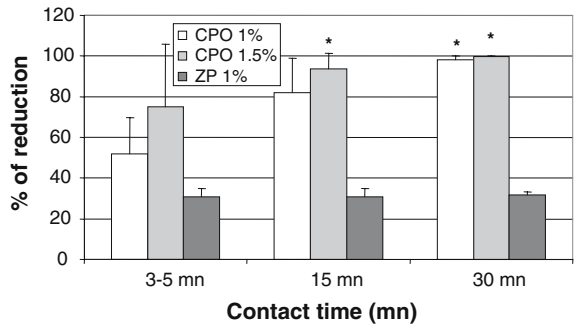

Figure 2. Evolution of the reduction percentage in viable counts (mean ± standard error) of *Malassezia restricta* IP 2392.96 according to the contact time (min) with the 1% and 1.5% ciclopiroxolamine solutions and the 1% zinc pyrithione solution. $*P < 0.05$ in comparison with the 3–5 min contact time.

antimicrobial mechanism described is the high affinity of CPO for trivalent metal cations [24] acting as cofactors for enzymes such as cytochromes involved in mitochondrial electron transport. At the same time, catalase and peroxydase activities are strongly inhibited by the drug, leading to a lethal lack of intracellular degradation of toxic peroxides [25]. We have recently reported the fungicidal efficacy of ciclopirox against dermatophytes, moulds and yeast strains on the basis of numeration of residual viable cells and electron microscopy observations [29]. The present study confirms such a fungicidal efficacy, in comparison to ketoconazole, which only acts as an inhibitory compound [30]. Above all, it emphasizes the time-dependent and dose-dependent effect of ciclopirox recently demonstrated *in vivo* by Altmeyer and Hoffmann [31].

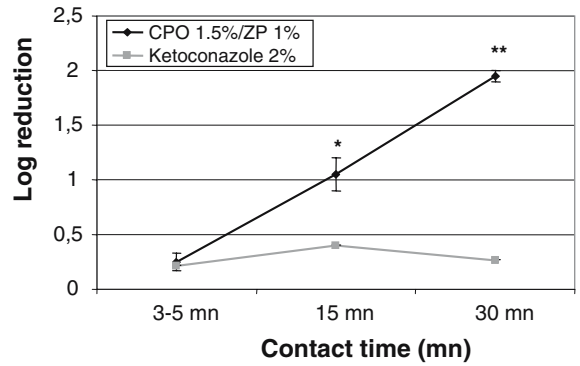

Figure 3. Evolution of the log reduction in viable counts (mean ± standard error) of *Malassezia globosa* IP 2387.96 according to the contact time (min) with the 1.5% ciclopiroxolamine/1% zinc pyrithione solution and the 2% ketoconazole solution. $*P < 0.05$, 2% ketoconazole versus 1.5% ciclopiroxolamine/1% zinc pyrithione. $**P < 0.01$, 2% ketoconazole versus 1.5% ciclopiroxolamine/1% zinc pyrithione.

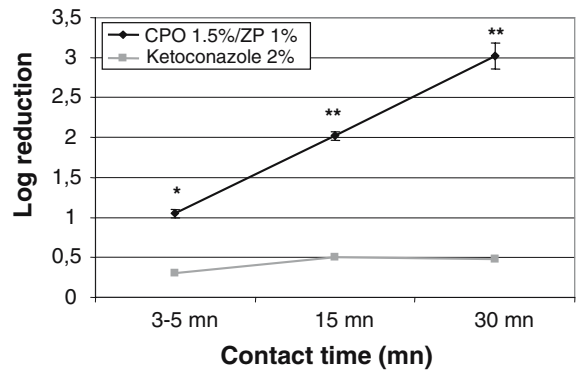

Figure 4. Evolution of the log reduction in viable counts (mean ± standard error) of *Malassezia restricta* IP 2392.96 according to the contact time (min) with the 1.5% ciclopiroxolamine/1% zinc pyrithione and 2% ketoconazole solutions. $*P < 0.05$, 2% ketoconazole versus 1.5% ciclopiroxolamine/1% zinc pyrithione. $**P < 0.01$, 2% ketoconazole versus 1.5% ciclopiroxolamine/1% zinc pyrithione.

Among the potent active compounds used in SD/dandruff, zinc pyrithione is also well recognized [11, 18, 19] despite the fact that its mode of action has not yet been clearly demonstrated. In our study, we report for the first time the highly potent inhibitory activity of this drug against *M. globosa* and *M. restricta*, similar to that of ketoconazole. The real antifungal efficacy of ZP must be considered in addition to its other properties such as the improvement of the altered stratum corneum ultrastructure observed in dandruff and SD [32]. These results must be confirmed

on other *Malassezia* species according to their prevalence in SD patients [33].

As underlined by Bulmer and Bulmer [34], few studies experimentally comparing the antifungal efficacy of current therapeutic shampoos have been carried out. Furthermore, the antifungal activity of the shampoos is generally evaluated *in vitro* by determining minimal inhibitory concentrations [30], which only reflect the fungistatic effect of the products. However, the conditions of use of these products, i.e. the application of high doses of shampoo for a short time, require specific *in vitro* analyses as previously noticed by Mayser et al. [35], and in particular the evaluation of their fungicidal activity. Moreover, when the fungicidal activity of the products is evaluated, the omission in the experiments of a validated end-of-contact step as described in the standards [23], often leads to false conclusions on the killing effect of antifungal agents [34]. Our study was performed to better define the antifungal efficacy of some antimycotic agents against *Malassezia* species, especially in terms of fungicidal effect, taking into account that the recurrent symptoms are frequently due to recolonization. Therefore, we evaluated the efficacy of CPO/ZP combination not only at controlling the growth of *Malassezia* species but also at reducing their population, using validated methods. Furthermore, we confirmed the compatibility of the two active compounds in this association, which produced a fungicidal and highly synergistic growth inhibitory effect against *Malassezia* strains.

Many active compounds have been evaluated in the past in seborrheic dermatitis treatment. Concerning CPO-based shampoos [14], numerous recent clinical studies have demonstrated their efficacy in the treatment of cutaneous affections like dandruff and scalp SD, CPO being used alone [15–17] or combined with other active substances [20]. Regarding the associations containing ZP and current antimycotic drugs, their interest has also been demonstrated in an *in vivo* study that evaluated the efficacy of a shampoo combining 2% ketoconazole and 1% ZP at improving dandruff [21].

Therefore, a therapeutic shampoo containing 1.5% CPO and 1% ZP could be a promising candidate for the treatment of scalp SD, taking into account the main properties of the 2 compounds, i.e. the fungicidal efficacy of CPO and the high inhibitory activity of ZP. Our results suggest that, besides the anti-inflammatory properties of CPO and ZP, the advantage to add a fungicidal agent to an inhibitory compound in a shampoo formulation would be the regular reduction in the cutaneous *Malassezia* counts, leading to a lower recolonization and finally, to a lower risk of recurrence. This is in accordance with a recent clinical study carried out by Pierard-Franchimont et al. [22] demonstrating that a 4-week treatment with a shampoo containing 0.75% piroctone olamine, a hydroxypyridone compound, associated with 2% salicylic acid and 0.5% elubiol led to a more significant reduction in *Malassezia* spp. counts than a coal tar shampoo, with a positive impact on the post-treatment phase.

Our *in vitro* results underscore the potential interest of the CPO/ZP association in the treatment of SD of the scalp, due to its highly synergistic effect on the growth control and the reduction of *Malassezia* population. However, further comparative double blind studies are needed to evaluate its clinical activity [36].

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References

1. Gupta AK, Bluhm R. Seborrheic dermatitis. J Eur Acad Dermatol Venereol 2004; 18: 13–26.
2. Gupta AK, Batra R, Bluhm R et al. Skin diseases associated with *Malassezia* species. J Am Acad Dermatol 2004; 785–798.
3. Gupta AK, Nicol K. The use of sulphur in dermatology. J Drugs Dermatol 2004; 3: 427–431.
4. Skinner RB, Noah PW, Taylor RM et al. Double blind treatment of seborrheic dermatitis with 2% ketoconazole cream. J Am Acad Dermatol 1985; 12: 852–856.
5. Pierard-Franchimont C, Hermanns JF, Degreef H et al. From axioms to new insights into dandruff. Dermatology 2000; 200: 93–98.
6. Nakabayashi A, Sei Y, Guillot J. Identification of *Malassezia* species isolated from patients with seborrhoeic dermatitis, atopic dermatitis, pityriasis versicolor and normal subjects. Med Mycol 2000; 38: 337–341.
7. Faergemann J Atopic dermatitis and fungi. Clin Microbiol Rev 2002; 15: 545–563.
8. Makimura K, Tamura Y, Kudo M et al. Species identification and strain typing of *Malassezia* species stock strains and clinical isolates based on the DNA sequences

of nuclear ribosomal internal transcribed spacer 1 regions. J Med Microbiol 2000; 49: 29–35.
9. Sugita T, Suto H, Unno T et al. Molecular analysis of molecular microflora on the skin of atopic dermatitis patients and healthy subjects. J Clin Microbiol 2001; 39: 3486–3490.
10. Gemmer CM, De Angelis YM, Theelen B et al. Fast, non-invasive method for molecular detection and differentiation of *Malassezia* yeast species on human skin and application of the method to dandruff microbiology. J Clin Microbiol 2002; 40: 3350–3357.
11. Johnson BA, Nunley JR. Treatment of seborrheic dermatitis. Am Fam Physician 2000; 61: 2703–2710.
12. Peter RU, Richarz-Barthauer U. Successful treatment and prophylaxis of scalp seborrhoeic dermatitis and dandruff with 2% ketoconazole shampoo: results of a multicentre, double-blind, placebo-controlled trial. Br J Dermatol 1995; 132: 441–445.
13. Pierard-Franchimont C, Pierard GE, Arrese JE, De Doncker P Effect of ketoconazole 1% and 2% shampoos on severe dandruff and seborrhoeic dermatitis: clinical, squamometric and mycological assessments. Dermatology 2001; 202: 171–176.
14. Gupta AK, Bluhm R. Ciclopirox shampoo for treating seborrheic dermatitis. Skin Therapy Lett 2004; 9: 4–5.
15. Lee JH, Eunc HC, Cho KH. Successful treatment of dandruff with 1.5% ciclopiroxolamine shampoo in Korea. J Dermatolog Treat 2003; 14: 212–214.
16. Abeck D. Rational of frequency of use of ciclopirox 1% shampoo in the treatment of seborrheic dermatitis: Results of a double-blind, placebo-controlled study comparing the efficacy of once, twice, and three times weekly usage. Int J Dermatol 2004; 43: S13–S16.
17. Lebwohl M, Plott T. Safety and efficacy of ciclopirox 1% shampoo for the treatment of seborrheic dermatitis of the scalp in the US population : Results of a double-blind, vehicle-controlled trial. Int J Dermatol 2004; 43: S17–S20.
18. Pierard-Franchimont C, Goffin V, Decroix J et al. A multicenter randomized trial of ketoconazole 2% and zinc pyrithione 1% shampoos in severe dandruff and seborrheic dermatitis. Skin Pharmacol Appl Skin Physio 2002; l15: 434–441.
19. Marks R, Pearse AD, Walker AP. The effects of a shampoo containing zinc pyrithione on the control of dandruff. Br J Dermatol 1985; 112: 415–422.
20. Squire RA, Goode K A randomised, single-blind, single-centre clinical trial to evaluate comparative clinical efficacy of shampoos containing ciclopiroxolamine (1.5%) and salicyclic acid (3%), or ketoconazole (Nizoral®) for the treatment of dandruff/seborrhoeic dermatitis. J Dermatolog Treat 2002; 13: 51–60.
21. Saple DG, Ravichandran G, Desai A. Evaluation of safety and efficacy of ketoconazole 2% and zinc pyrithione 1% shampoo in patients with moderate to severe dandruff – a postmarketing study. J Indian Med Assoc 2000; 98: 810–811.
22. Pierard-Franchimont C, Pierard GE, Vroome V et al. Comparative anti-dandruff efficacy between a tar and a non-tar shampoo. Dermatology 2000; 200: 181–184.
23. AFNOR. Recueil des normes "Antiseptiques et désinfectants" 1991.
24. Markus A. Hydroxy-pyridones: Outstanding biological properties. In: Shuster S., ed. Hydroxy-pyridones as antifungal agents with special emphasis on onychomycosis, SpringerBerlin, 1999: 1–10.
25. Reitze HK, Seitz KA, Dannhorn DR. Enzyme histochemical investigations of *Candida albicans* after treatment with rilopirox, a novel fungicidal hydroxyl-pyridone. In: Shuster S., ed. Hydroxy-pyridones as antifungal agents with special emphasis on onychomycosis, SpringerBerlin, 1999: 11–17.
26. Niewerth M, Schaller M, Korting HC, Hube B. Mode of action of ciclopiroxolamine on *Candida albicans*. Mycoses 2002; 45: 63–68.
27. Jue SG, Dawson GW, Brogden RN Ciclopirox Olamine 1% cream. A preliminary review of its antimicrobial activity and therapeutic use. Drugs 1985; 29: 330–341.
28. Hanel H, Smith-Kurtz E, Pastowsky S. Therapy of seborrheic eczema with an antifungal agent with an antiphlogistic effect. Mycoses 34 suppl 1991; 1: 91–93.
29. Jomard P, Luc J, Roques C. *In vitro* fungicidal efficacy of ciclopirox. J Mycol Med 2004; 14: 78–82.
30. Velegraki A, Alexopoulos EC, Kriticou S et al. Use of fatty acid RPMI 1640 media for testing susceptibilities of eight *Malassezia* species to the new triazole posaconazole and to six established antifungal agents by a modified NCCLS M27-A2 microdilution method and Etest. J Clin Microbiol 2004; 42: 3589–3593.
31. Altemeyer P, Hoffmann K. Efficacy of different concentrations of ciclopirox shampoo for the treatment of seborrheic dermatitis of the scalp: Results of a randomized, double-blind, vehicle-controlled trial. Int J Dermatol 2004; 43: S9–S12.
32. Warner RR, Schwartz JR et al. Dandruff has an altered stratum corneum ultrastructure that is improved with zinc pyrithione shampoo. J Am Acad Dermatol 2001; 45: 897–903.
33. Sandtröm Falk MH, Tengvall Linder M, Johansson C, Bartosik J, Bäck O, Särnhult T, Wahlgren CF, Scheynius A, Faergemann J. The prevalence of *Malassezia* yeasts in patients with atopic dermatitis, seborrhoeic dermatitis and healthy controls. Acta Derm Venereol 2005; 85: 17–23.
34. Bulmer AC, Bulmer GS The antifungal action of dandruff shampoos. Mycopathologia 1999; 147: 63–65.
35. Mayser P, Argembeaux H, Rippke F. The hair strand test – a new method for testing antifungal effects of antidandruff preparations. J Cosmet Sci 2003; 54: 263–270.
36. Lorette G, Ermosilla V. Clinical efficacy of a new ciclopiroxolamine/zinc pyrithione shampoo in scalp seborrheic dermatitis treatment. Eur J Dermatol. 2006; 16:1–7.

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