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Evaluation of topical antifungal products in an *in vitro* onychomycosis model

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SUMMARY

Many topical commercial products are currently available for the treatment of onychomycosis. However, limited data are available concerning their antifungal activity. Using an *in vitro* onychomycosis model, the daily application of seven nail formulations was compared to the antifungal reference drug amorolfine (Loceryl®) and evaluated for inhibitory activity against *Trichophyton mentagrophytes* using an agar diffusion test. Of all commercial nail formulations, only Excilor® and Nailner® demonstrated inhibitory activity, which was much lower compared to the daily application of Loceryl®. However, Excilor® showed similar efficacy compared to the conventional weekly application of Loceryl®. These results suggest a role for organic acids in the antifungal effect of Excilor® (acetic acid, ethyl lactate) and Nailner® (lactic acid, citric acid, ethyl lactate) as all tested formulations without organic acids were inactive.

KEYWORDS: onychomycosis, *Trichophyton mentagrophytes*, topical antifungals

Introduction

Although onychomycosis used to be regarded as a purely aesthetic problem, it is currently considered as a disease, as it can have a serious impact on a patient's quality of life, resulting in physical and psychological discomfort [1; 2]. With up to 20% of the world population infected, onychomycosis should be considered as an important public health problem [3]. Since current oral antifungals may cause liver toxicity and potential drug interactions, an increasing number of patients prefer the use of topical therapy. Over the last 10 years, few new topical antifungal drugs have been developed and introduced, particularly due to the poor permeability of the nail [4]. Instead, a large number of topical alternatives are commercially available to the consumer without prescription. These formulations often lack pharmacological substances and exert their effect by alternative modes of action such as (i) acidification of the nail, yielding an unfavourable environment for fungal growth (physical action), or (ii) by addition of natural or cosmetic ingredients with some antifungal activity [5; 6].

While these products claim to be very efficient, scientific proof of activity against onychomycosis is rather limited. In the present study, we examined the activity of seven European commercial products in an *in vitro* onychomycosis model using *Trichophyton mentagrophytes*, and compared their activity to the topical antifungal drug amorolfine (Loceryl®).

Materials and methods

Experimental formulations

Excilor® (Oystershell, Belgium), Nailner Nail Fungus pen® (YouMedical, The Netherlands), Naloc Nail Treatment® (Meda Pharma, Sweden), Mycosan® (Mycosan, The Netherlands), Boots Fungal Nail Treatment pen® (Boots UK Limited, United Kingdom), Kruidvat fungal

nail pen® (Kruidvat BVBA, Belgium) and Scholl Fungal Nail Treatment® (Reckitt Benckiser, United Kingdom) were compared to the most popular medicinal product Loceryl nail polish® (50 mg/ml amorolfine) (Galderma, Switzerland) for antifungal activity in an *in vitro* onychomycosis model. Test products were obtained either by collection of liquid or lacquer from the bottle or from the donor compartment of the pen.

Methodology

To determine the antifungal activity of the nail formulations, an *in vitro* onychomycosis model was used as previously described [7]. Briefly, mounted bovine hoof slices with approximate thickness of 600 µm were placed on a non-inoculated agar. Twenty µl of test solution was added to the surface of the hoof slices daily for 7 consecutive days to preload the hoof. After preincubation, the slices were transferred to a freshly inoculated *T. mentagrophytes* B70554 (Scientific Institute of Public Health, Brussels, Belgium) (10^5 CFU) agar and test solutions were applied daily for another 7 days. Because Loceryl® nail lacquer is not authorised for daily application and renewal in patients, we also included conventional, weekly application and renewal of Loceryl®. During the whole experiment, agars were incubated at 27°C and the inhibition zone was calculated at 7 days post-inoculation as the area of inhibition relative to the total area of growth (vehicle) using ImageJ 1.48 software (Java-based freeware for advanced image processing). As the activity of some tested formulations relies on decreasing the pH of the fungal environment, minimal buffered agars containing only deionised water, dextrose, $MgSO_4$ and casein hydrolysate (Sigma-Aldrich, Belgium) were used.

Statistical analysis

Statistical analysis was performed with GraphPad Prism V.6.0 using One-way ANOVA. P-values below 0.05 were considered significant. Reported values are expressed as mean ± SD.

Results

Using an *in vitro* nail permeability model on bovine hoof slices, daily application of seven marketed nail formulations was compared to daily as well as weekly application of the standard antifungal drug Loceryl®. Only Excilor® and Nailner® inhibited fungal growth showing respectively 4.6 ± 1.5% and 1.1 ± 0.5% inhibition (**Figure 1**) which was rather low compared to daily dosing of Loceryl® (24.5 ± 5.4% inhibition). However, Excilor® demonstrated similar antifungal activity in comparison to weekly dosing of Loceryl® (3.2 ± 4.4% inhibition). In contrast, Naloc®, Mycosan®, Boots®, Scholl® and Kruidvat® showed no activity against *T. mentagrophytes*.

Discussion

All tested commercial products are substance-based medical devices that claim a physical action and are sold in Europe for the treatment of fungal nail infections. Their activity was tested against Loceryl® containing 5% amorolfine (MIC = 0.32 µg/ml) [7]. Mycosten® (8% ciclopirox lacquer), another potential reference drug, was not used because of its lack of activity in the onychomycosis model (data not shown). This can be explained by the much higher MIC of its antifungal compound ciclopirox (MIC = 2.49 µg/ml).

To clarify the results of the tested products, a summary of their main ingredients is given in **Tables 1 and 2**, where products are categorised according to their active ingredient content, namely products containing organic acids and products with antimicrobial natural/cosmetic ingredients. The activity of Excilor® is likely based on the combination of acetic acid and ethyl lactate, which dissociates in lactic acid and ethanol, together with a decrease of pH

underneath the nail plate. While antifungal activity of these organic acids has never been confirmed against dermatophytes, activity against other fungi was demonstrated [8; 9]. Furthermore, the combination of multiple organic acids could have a synergistic effect [9; 10]. Importantly, at the level of the fungal cells, only the undissociated acid is able to diffuse through the cell membrane. Once inside the cell, dissociation of the acid will lead to intracellular acidification and anion accumulation causing inhibition of metabolic activity and apoptosis [8]. By decreasing the extracellular pH, a higher concentration of undissociated acid will be available to diffuse through the cell membrane, thus facilitating antifungal activity [9]. Another formulation with slight activity against *T. mentagrophytes* was Nailner®, containing lactic acid, citric acid and ethyl lactate as main ingredients. Its lower activity compared to Excilor® may be explained by the lower intrinsic activity of lactic acid and citric acid compared to acetic acid [9]. Besides urea and lactic acid, Naloc® consists of propylene glycol which acts against *Trichophyton* species by disrupting the cell wall and causing the cells to collapse by osmotic changes [11; 12]. In a 24-week double-blind placebo-controlled study, daily treatment with urea, lactic acid and propylene glycol demonstrated to be effective against distal subungual onychomycosis [5]. However, in our study we were unable to demonstrate a significant antifungal effect after two weeks of treatment with Naloc®. Mycosan®, Boots® and Kruidvat® all contain pentylene glycol, which is more active against *Trichophyton* species *in vitro* [13], but did not demonstrate any growth inhibition in our onychomycosis model. Finally, no activity was found for Scholl®, the only product for which no ingredients are mentioned in the patient information leaflet, making it impossible to clarify its action.

In summary, Loceryl® demonstrated superior activity compared to all other formulations when applied and renewed daily. One organic acid and ester-based product (Excilor®), demonstrated similar activity when compared to the clinically prescribed weekly application

of Loceryl®. Although Naloc® wasn't able to cause any fungal growth inhibition, our results suggest a substantial role for organic acids in the antifungal effect of both Excilor® (acetic acid, ethyl lactate) and Nailner® (lactic acid, citric acid, ethyl lactate) since all products without organic acids were inactive. Moreover, these findings support the claim that some commercial products are able to penetrate the nail and have an antifungal effect. As antifungal treatment is rather trivial in most onychomycosis cases and remains expensive, patients may consider these options as a first line treatment.

Acknowledgements

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Conflict of interest

The study was performed on a product (Excilor®) of the company Oystershell NV who partially funded this research. The terms of this funding have been reviewed and approved by the University of Antwerp in accordance with its policy on objectivity in research.

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175 **Table 1:** Main ingredients of nail formulations, containing organic acids.

	Excilor®	**Nailner®**	**Naloc®**
Activity	Acetic acid Ethyl lactate Water	Lactic acid Citric acid Ethyl lactate	Lactic acid Propylene Glycol
Penetration enhancer	Dimethyl isosorbide	-	Urea
pH	4.2	1.3	5.4

176 ** Scholl was not included in both tables, because of the lack of product description in the leaflet.*

177 **Table 2:** Main ingredients of nail formulations, using natural/cosmetic ingredients.

	Mycosan®	**Boots®**	**Kruidvat®**
Activity	*Lactobacillus* Rye ferment filtrate Pentylene glycol	Olive-leaf oil Pentylene glycol	*Melaleuca alternifolia* leaf oil Pentylene glycol
Penetration enhancer	Dimethyl isosorbide	Dimethyl isosorbide	
pH	4.2	5.8	8.5

178 * *Scholl was not included in both tables, because of the lack of product description in the leaflet*

179 **Figure 1:** Biological activity of Excilor®, Nailner® and reference drug Loceryl® and the absence of activity of
180 vehicle, Naloc®, Mycosan®, Boots®, Kruidvat®, and Scholl® against T. mentagrophytes after 7 days of
181 incubation (2 independent experiments, 3 repeats).

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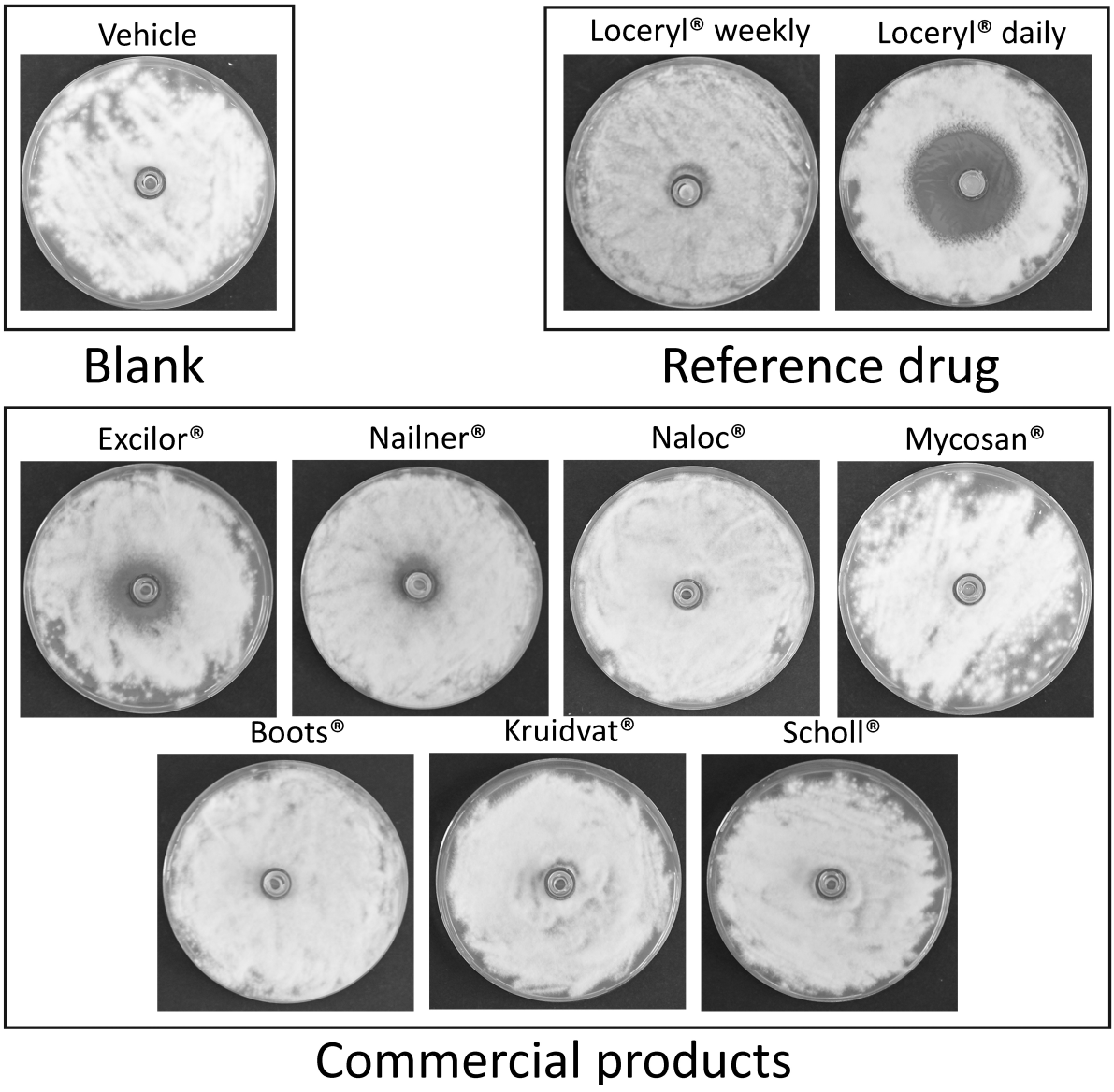

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