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Original

Combined activity of itraconazole and terbinafine on clinical isolates of *Neoscytalidium dimidiatum*

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Abstract

Aim: Aim: to analyze the susceptibility of N. dimidiatum to the combined effect of itraconazole and terbinafine.

Methods: The Minimum Inhibitory Concentration and Fractional Inhibitory Concentration were determined *in vitro* by the chessboard method for 15 clinical isolates of onychomycosis, from different patients, all positive for *N. dimidiatum*. Duplicate trials were prepared with combined dilutions of antifungals and the effect of both drugs was evaluated.

Results: The average Minimum Inhibitory Concentration of Itraconazole when applied alone for the isolates was 30.83 µg/mL and 4.49 µg/mL when combined with Terbinafine. The average Minimum Inhibitory Concentration of Terbinafine alone was 0.33 µg/mL and 0.07 µg/mL when combined with Itraconazole. Statistically significant differences were found between the average Minimum Inhibitory Concentrations of the antifungals analyzed alone versus the Minimum Inhibitory Concentrations obtained by mixing both compounds. That is for Itraconazole (t = 2,958; gl = 14; p = 0,01) and (t = 4,721; gl = 14; p <0,001) for Terbinafine. Combined use showed 40 % synergism.

Conclusion: The Itraconazole-Terbinafine combination had a synergistic effect to inhibit the growth of *N. dimidiatum,* which offers a therapeutic alternative in the treatment of onychomycoses caused by this fungus.

Keywords: Onychomycosis, Itraconazole, Terbinafine, Combined Modality Therapy.

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Abbreviations: ASG, Sabouraud glucose agar; FIC, fractional inhibitory concentration; MIC, minimum inhibitory concentration.

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Neoscytalidium dimidiatum is a non-dermatophyte filamentous ascomycete of medical importance [1-4] that since 1970[5,6] has been described as a causative agent of disseminated infections, onychomycosis, and other clinical manifestations in humans. [1,7-14] Since the 1990s *N. dimidiatum* has been found in the Americas.[8,15,16] It is acquired from soil or plant matter by direct contact or trauma, [3,17]although some authors such as Moore (1986) and Campbell (1971) suggest the possibility of person-to-person transmission.[12,18] It is a primary pathogen thanks to its keratolytic capacity (keratinases, lipases, and amylases hydrolyze the keratin of skin or nails to facilitate its entry) and its pigment melanin, as a virulence factor, protects it against the action of the host immune system.[3,12,15,16,18,19] Onychomycosis is a highly prevalent mycosis worldwide.[20,21] In Costa Rica these infections have been estimated between (16 - 24) %, where *N. dimidiatum* appears as the cause of onychomycosis in 2.8 % in toenails and 4.8 % in fingernails.[22,23] *N. dimidiatum* generally enters through the nail bed and distal lateral folds. Once installed it generates onychodystrophy, with whitish depigmentation and onycholysis. With time, koilonychia, subungual hyperkeratosis, yellowish-brownish-blackish pigmentation and if the infection reaches the proximal border, paronychia may develop.[8,24] In immunosuppressed patients, whether due to transplantation (solid organ or bone marrow), systemic lupus erythematosus, rheumatoid arthritis, long-term corticosteroid use, human immunodeficiency virus (HIV) infection, diabetes mellitus, cirrhosis or being older than 60 years risk factors, can lead to systemic infection with a mortality of up to 50%. [10-12,25-27] Regarding the treatment of onychomycosis, in clinical practice *N. dimidiatum* has shown resistance to many azole antifungal agents, allylamine derivatives, morpholines, and ciclopirox [28]and currently no effective treatment protocol has been standardized. [22,23,29- 31] Studies such as that of Lacroix and Chauvin (2008) have analyzed the *in vitro* susceptibility of this fungus against different antifungal agents, from which they report minimum inhibitory concentrations (MIC) tested alone of 0.25 µg/mL for voriconazole; 0.50 µg/mL for amphotericin B; 0.50 µg/mL for terbinafine; 2 µg/mL for posaconazole; 8 µg/mL for caspofungin and >16 µg/mL for itraconazole. [24,29] However, to date, no such study has been reported in the literature in which the combined effect of antifungals on the fungus in question is determined. Therefore, the present investigation aimed to determine the combined *in vitro* interaction of itraconazole and terbinafine on clinical isolates of *N. dimidiatum-positive* onychomycosis.

Methods

Isolations: 15 isolates of *N. dimidiatum* obtained from different patients diagnosed with onychomycosis and deposited in the Mycotheque of the Faculty of Microbiology, University of Costa Rica, between 2009 and 2016 were analyzed. The fungi were cultured in tubes with Sabouraud glucose agar (ASG) at room temperature (25 - 35) °C. Prior to the susceptibility analyses, their colonial and microscopic morphology (in clear lactophenol) was analyzed to verify that phenotypically the isolates corresponded to *N. dimidiatum*. [32]

Checkerboard method: The minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) were determined by the checkerboard method.[33,34] All cultures and assays were performed in duplicate. For this purpose, a stock solution of each antifungal (terbinafine 6400 µg/mL and itraconazole 1600 µg/mL) (Royal Pharm, Hangzhou, China) was prepared using dimethyl sulfoxide (DMSO) (Sigma Chemicals Co., St. Louis, Mo, USA) as a diluent. From the stock solution, serial twofold dilutions were made in RPMI medium (*Roswell Park Memorial Institute*) and labeled as follows: A1 to A8 for terbinafine and B1 to B8 for itraconazole. For the scheme of microtitre plate filling Figure 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A			B1+A1	B2+A1	B3+A1	B4+A1	B5+A1	B6+A1	B7+A1	A2		
B			B1+A2	B2+A2	B3+A2	B4+A2	B5+A2	B6+A2	B7+A2	A3		
C			B1+A3	B2+A3	B3+A3	B4+A3	B5+A3	B6+A3	B7+A3	A4		
D			B1+A4	B2+A4	B3+A4	B4+A4	B5+A4	B6+A4	B7+A4	A5		
E			B1+A5	B2+A5	B3+A5	B4+A5	B5+A5	B6+A5	B7+A5	A6		
F			B1+A6	B2+A6	B3+A6	B4+A6	B5+A6	B6+A6	B7+A6	A7		
G			B1+A7	B2+A7	B3+A7	B4+A7	B5+A7	B6+A7	B7+A7	A8		
H			B2	B3	B4	B5	B6	B7	B8	CC	BR	

Figure 1. Distribution of antifungals for the determination of the fractional inhibitory concentration by the Checkerboard method in the 96 hole microplate (A: terbinafine, B: itraconazole, CC: growth control and BR: reagent blank).

Inoculum preparation: ASG isolates were seeded with aseptic technique in potato dextrose agar (APD) and incubated for 7 days at room temperature, to favor sporulation. Each suspension of *N. dimidiatum* arthrospores was prepared from APD in 0.85 % saline using a Bürker chamber (Poly-Optik GmbH, Blankemburg, Germany) and adjusted to a concentration of (1 - 5) x10 ^{6}arthrospores/mL. It was then diluted 1:50 in RPMI medium. Microtiter wells were inoculated with 100 µL of the spore suspension. The plates were incubated at room temperature without shaking for 72 hours until growth was obtained in the growth control (GC) well.

MIC determination: MIC was determined as the lowest concentration that produced 80% inhibition of growth, when compared against the CC. Spectrophotometric reading (λ = 450 nm) was performed with a Synergy HT plate reader (BioTek Instruments, Inc.; Winooski, VT, USA).

Determination of the IPC: The absorbance of the medium (i.e. the absorbance of well 11H) was subtracted from each well. The CIF value (or CIF index) was calculated based on the following **equation:** CIF index = $CIF_A/CMI_A + CIF_B/CMI_B$, where CIF_A is the MIC of drug A in combination and MIC_A is the MIC of drug A alone; CIF_B is the MIC of drug B in combination and MIC is the $_B$MIC of drug B alone. The CIF index is based on the hypothesis that a drug cannot interact with itself and therefore the effect of the combination if CIF is ⩽ 0.5 is considered synergism; if CIF > 0.5 - < 4.0 it means no effect and finally when CIF is ⩾ 4.0 it means antagonism. [34-40]

$$CIF\ Index = CIF_A/CMI_A + CIF_B/CMI_B \textbf{(formula 1)}$$

Equation A-1 for the calculation of the fractional inhibitory concentration.

Statistical analysis: Results were analyzed using SPSS for Windows version 20 (SPSS Inc., Chicago, Illinois, USA). Geometric mean, $MIC_{50,}$ and MIC_{90} percentiles were estimated where terbinafine and itraconazole inhibited fungal multiplication. Subsequently, a *t-student* analysis was performed to determine whether statistically significant differences exist between concentrations alone and concentrations in combination.

Results

Clinical isolates: all 15 isolates exhibited the typical colonial morphology corresponding to fungi of the species *N. dimidiatum*. Mounts of all isolates in clear lactophenol showed the presence of septate fuliginous mycelium and arthrospores (Figure 2).

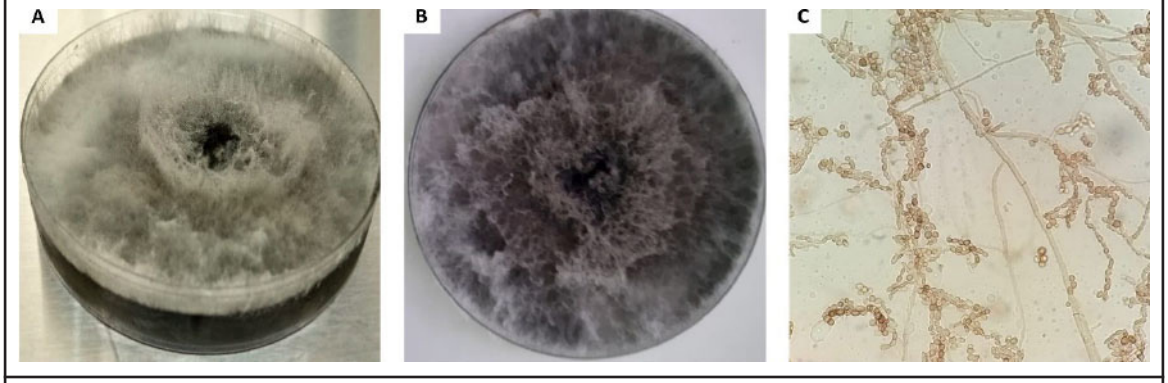

Figure 2. Cultures (A and B) and microscopic morphology of *N. dimidiatum (*C).

Determination of *in vitro* susceptibility patterns: based on the tests performed, MICs (Table 1) and IPCs of clinical isolates of *N. dimidiatum* were estimated. The antifungals used were itraconazole and terbinafine, tested individually and in combination. Table 2 shows the susceptibility patterns of each antifungal test. Statistically significant differences were found between the MIC means when comparing itraconazole used alone to itraconazole combined with terbinafine (t = 2.958; gl = 14; p = 0.01). Also, between the means of MICs of terbinafine used alone and terbinafine combined with itraconazole (t = 4.721, Gl = 14, P < 0.001). Lower MICs were obtained when combining both drugs. In the case of itraconazole 93.33 % (*n* = 14) of the isolates showed resistance (MIC ⩾ 1 µg/mL) [41] when its effect was evaluated when applied alone, but when combined with allylamine this percentage decreased to 60.00 % (*n* = 9) (Table 1).

Table 1. *In vitro* activity of terbinafine and itraconazole combined or alone, applied on clinical isolates of *N. dimidiatum (*n = 15)

Fungus	***CMIItraconazole (µg/mL)**	**CMIItraconazole combined** (µg/mL)	****CMITerbinafine** (µg/mL)	**CMITerbinafine combined** (µg/mL)
NEO SCY 03	64,00	4,00	0,50	0,13
NEO SCY 05	64,00	2,00	0,50	0,25
NEO SCY 06	64,00	4,00	0,50	0,13
NEO SCY 09	0,40	0,20	0,13	0,02
NEO SCY 10	1,00	0,50	0,13	0,01
NEO SCY 11	1,00	0,50	0,13	0,03
NEO SCY 12	4,00	2,00	0,13	0,02
NEO SCY 14	32,00	16,00	0,13	0,03
NEO SCY 16	64,00	16,00	0,25	0,02
NEO SCY 17	2,00	0,50	0,52	0,07
NEO SCY 18	1,50	0,38	0,13	0,02
NEO SCY 19	128,00	16,00	0,25	0,03
NEO SCY 21	3,00	1,50	0,50	0,03
NEO SCY 22	1,60	0,80	1,00	0,13
NEO SCY 23	32,00	2,00	0,13	0,06

Table 2. Distribution of minimum inhibitory concentration (MIC) of clinical isolates of *N. dimidiatum* (n = 15) using itraconazole and terbinafine as antifungal agents

Antifungal	MIC (µg/mL)			
	Average (SD)	Range	WCC_{50}	WCC_{90}
Itraconazole	30,83 ± 38,35	0,40 - 128,00	3,00	64,00
Itraconazole combined*	4,49 ± 6,07	0,20 - 16,00	2,00	16,00
Terbinafine	0,33 ± 0,25	0,13 - 1,00	0,13	00,50
Terbinafine combined**	0,07 ± 0,07	0,01 - 0,25	0,03	00,13

*Combination of itraconazole and terbinafine
**Combination of terbinafine with itraconazole

The average CIF of both antifungals combined was 0.59 µg/mL required to inhibit fungal multiplication. The net result of mixing the antifungals evidenced 40% (*n* = 6) synergism in the cases studied (CIF ⩽ 0.5). There were no cases of antagonism in the isolates studied (Figure 3).

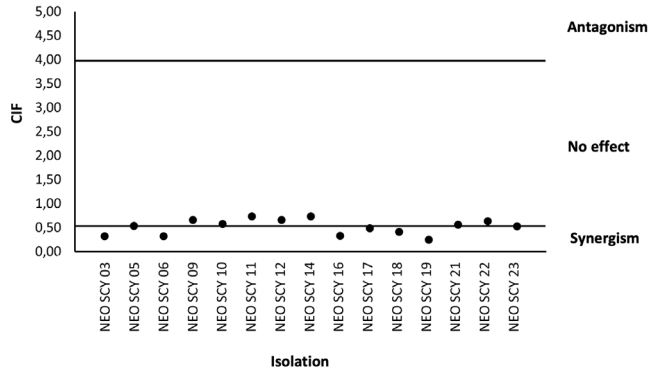

Figura 3. Effect of fractional inhibitory concentration (FIC) of terbinafine and itraconazole combined on clinical isolates of *N. dimidiatum (*n=15).

Discussion

Although onychomycosis caused by *N. dimidiatum* can be considered a therapeutic challenge,[23,29-31] the present work tested the joint effect of itraconazole and terbinafine on 15 isolates of the fungus, in order to find an alternative treatment.

In the present investigation, when terbinafine was applied alone, a MIC between (0.13 - 1.00) µg/mL was found, which is in agreement with those reported in countries such as England, France, Spain, Canada, Colombia, and Brazil where the range of MIC reported is (0.03 - 4.00) µg/mL.[29,42-46] The variability in MIC values may be due to the fact that the isolates come from different strains and therefore exhibit different susceptibilities to this allylamine. The *in vitro* results could correlate with the fact that *in vivo* terbinafine is very rapidly absorbed independent of acidity, reaches peak values at two hours post-ingestion, is highly keratinolytic, and is lipophilic, fungicidal, and does not undergo first-pass metabolism. [47-50]

In the present work, the MIC_{50} of terbinafine used alone was 0.13 µg/mL and for MIC_{90} was 0.50 µg/mL. In the Netherlands, Dorsthorst *et al.* (2002) found an MIC_{50} of 4 µg/mL of terbinafine alone to inhibit the growth of *Aspergillus fumigatus.*[33] In Spain, the study by Garcia *et al.* (2005) using the Sensititre YeastOne® microdilution technique estimated the MIC_{50} for *A. fumigatus* at 0.50 µg/mL.[51] In Colombia, using the E-test method, Chávez *et al.* (2010) found an MIC_{90} of 0.38 µg/mL to inhibit *A. fumigatus*.[52] In Costa Rica, Ramirez-Hernandez et al. (2020) by means of plate microdilution (CLSI) found an MIC_{50} of 0.50 µg/mL and MIC_{90} of 1.36 µg/mL to disrupt the growth of *Aspergillus versicolor*.[53] This allows inferring that *N. dimidiatum* requires lower concentrations of terbinafine alone than *Aspergillus* sp. to inhibit its multiplication. The results of other works including this one suggest terbinafine as an antifungal against *N. dimidiatum* since it was determined that this allylamine used alone exerted a greater inhibitory effect than azole. It is important to highlight that, results are compared between the genus *Aspergillus* sp. and *Neoscytalidium* sp. because both are filamentous fungi that cause onychodystrophies and can generate systemic infections in immunocompromised patients.

On the other hand, itraconazole analyzed alone presented a MIC between (0.40 - 128.00) µg/mL, which coincides with that reported in countries such as England, Holland, France, Belgium and Colombia where the range of MIC reported is (0.03 - >64.00) µg/mL.[24,29,38,42,44,54] The variants in the MICs of these studies derive from the fact that the isolates evaluated may belong to different strains and therefore present different resistances.

In the present investigation the MIC_{50} of itraconazole used alone was 3.00 µg/mL and for MIC_{90} was 64.00 µg/mL. In Colombia, Chavez *et al*. (2010) by E-test method found both MIC_{50} and MIC_{90} >1.00 µg/mL to inhibit *A. fumigatus*; MIC_{50} of 1.5 µg/mL and MIC_{90} of 3.00 µg/mL against *Aspergillus niger*; and both MIC_{50} and MIC_{90} at 0.50 µg/mL against *Aspergillus flavus*.[52] In Costa Rica, MIC_{50} of 1.00 µg/mL and MIC_{90} of 1.80 µg/mL were found to interrupt the growth of *A. versicolor,* by the CLSI broth microdilution technique.[53] In Spain, the study by Garcia *et al.* (2005) using Sensititre YeastOne® microdilution estimated MIC_{50} at 0.13 µg/mL for *A. fumigatus*; MIC_{50} at 0.50 µg/mL against *A. niger*; and MIC_{50} at 0.25 µg/mL against *A. flavus*.[51] In the Netherlands, Dorsthorst *et al.* (2002) determined the MIC_{50} at 0.25 µg/mL of itraconazole alone to stop the multiplication of *A. fumigatus*.[33] Results from other papers including this one demonstrate the requirement for higher concentrations of itraconazole used alone to inhibit the growth of *N. dimidiatum* and lower concentrations against *Aspergillus* sp. This suggests that itraconazole, when used alone, exerts a lower inhibitory effect on *N. dimidiatum*. These results for itraconazole alone *in vitro* could correlate *in vivo* with the fact that this drug is dose-dependent, its duodenal absorption requires an acidic pH (which is not easy to achieve because acidic gastric emptying induces alkalinization of the duodenum). Furthermore, a blood concentration of at least ⩾ 5 µg/mL is required to see any effect, which is also difficult because CYP3A4 performs a first-pass metabolism at the small intestine level, which reduces its bioavailability before it reaches the nail vascular bed to exert its fungistatic effect; there may also be intrinsic resistance of some isolates of the fungus to azoles. [41,47,49,55] In addition, the widespread use of azole antifungal drugs has been associated with the emergence of resistant or less sensitive species in many regions of the world and in specific patient populations. [56]

Regarding the effect of the combination of both treatments, in 2002, Dorsthorst et al. reported a synergistic effect of combining itraconazole and terbinafine on *A. fumigatus* isolates.[33] In the study of a fatal case of pulmonary aspergillosis, led by Meletiadis et al. (2010) (supported by the Intramural Research Program of the National Cancer Institute, Bethesda, Maryland, USA) and the study by Hall *et al*. (1983) in which terbinafine and itraconazole were analyzed, it was confirmed that even weak interactions with CIF between 0.5 and 0.99 proved to be statistically significant.[40,57] On the other hand, Ramirez-Hernandez and collaborators (2020) found a potentiating effect of terbinafine on itraconazole with isolates of *A. versicolor*. [53] The present study found not only this potentiating effect but also a 40% synergism.

The synergistic effect derives from the fact that terbinafine inhibits the enzyme squalene epoxidase (in the first step of ergosterol biosynthesis) and itraconazole inhibits 14-alpha-sterol demethylase (in the middle of the biosynthetic cycle). [47,55] This is a pharmacokinetic and pharmacodynamic advantage because at the hepatic level both drugs are metabolized by different pathways, there is no negative interaction or enzymatic saturation of the detoxifying cytochromes, which reduces the hepatic accumulation of these drugs. This results in a pharmacological benefit to the patient because their synergy when combined, and their rapid clearance, contribute to a lower likelihood of hepatocellular damage. [48,50,55,58-60] The net pharmacological action of combining itraconazole and terbinafine interrupts two key steps in the biosynthesis of ergosterol in the fungus; this weakens its membrane, facilitating the entry of these drugs into the fungus and exposing it to the action of leukocytes of the immune system. [55,59]

It is worth mentioning that when a patient receives therapies such as cyclosporine, sirolimus, tacrolimus, efavirenz, lovastatin, sinvastatin, atorvastatin, and fluvastatin, the competitive inhibition of itraconazole on CYP3A4 induces a plasma increase of these drugs, which leads to intoxications, the risk of rhabdomyolysis and myopathies.[50,55,59] Ritonavir, nelfinavir, cobicistat, darunavir or miconazole exert competitive inhibition on CYP2D6, so that terbinafine accumulates in the liver causing hepatopathies such as tissue necrosis. These negative interactions are enhanced with polymorphisms that produce homozygous slow metabolizers and intoxications

can result in the death of the patient.[50,58,60] Therefore, the physician should evaluate each case before administering therapy that includes itraconazole or terbinafine in immunosuppressed patients or those receiving antiretrovirals.

In conclusion, itraconazole monotherapy was not effective *in vitro* in eliminating *N. dimidiatum*. Terbinafine monotherapy was effective *in vitro* in inhibiting the fungus. On the other hand, the combination of itraconazole and terbinafine *in vitro* presented a total or partial synergistic action in inhibiting the growth of the fungus studied.

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