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Nanoemulsion Improves the Antifungal Activity of Allylic Thiocyanates against Yeasts and Filamentous Pathogenic Fungi

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We report the antifungal and antichemotactic activities of a series of allylic thiocyanates with low toxicity. We also show improved antifungal activity of the most promising compound when used in a nanoemulsion (NE). The 4-chlorophenyl-substituted allylic thiocyanate (compound **11**) exhibited a broad spectrum of antifungal activity and showed antichemotactic effects with 100% reduction in leucocyte migration. Minimal inhibitory concentrations ranged from 25 to 50 μg mL^{-1}, and the mechanism of action was related to complexation with fungal ergosterol. The NE containing compound **11** enhanced the antifungal activity approximately 64-fold for dermatophytes and 4-fold for *Candida* spp.. Compound **11** was not mutagenic and did not cause cell death or significant haemoloysis, although it exhibited mild dose-dependent DNA damage. It was not an irritant for chorioallantoic membrane of fertile white eggs and exhibited 100% inhibition of fungal growth in an *in vivo* model of dermatophytosis. Our data indicate that allylic thiocyanates are very promising for the antifungal potential in nanostructured systems, with associated anti-inflammatory effect.

Introduction

Fungal infections produce high rates of morbidity and mortality, especially in severely ill or immunocompromised patients.[1,2] *Candida albicans* is a prominent fungal pathogen in humans. It is responsible for a wide spectrum of clinical presentations, and infection that can lead to death.[3] In the last 20 years, *C. albicans* has been the most common strain isolated from hospitalized patients. However, non–*albicans Candida* (NAC) infections are rapidly growing. *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei* account for 95–97% of all invasive fungal infections caused by yeast of this genus.[4] In addition to yeast infections, fungal infections caused by dermatophytes have increased during the last decades.[5] These conditions also lead to morbidity-associated cutaneous mycoses that are frequently ineffectively treated.[6]

Illnesses caused by *Candida* spp. are associated with inflammatory processes (also observed for dermatophytoses)[7,8] that are possibly exacerbated by enzymes secreted by the fungus during invasion.[9] Therefore, uncontrolled inflammation can compromise treatment and lead to other associated diseases.[10,11] Chemical compounds have been investigated for their ability to inhibit leucocyte migration through an anti-inflammatory mechanism (antichemotactic activity).[12] Thus, it is interesting to investigate if new libraries of molecules exert antifungal and anti-inflammatory effects.

Although numerous effective antifungal agents are available, their therapeutic outcome is less than optimal due to limitations associated with toxicity and physicochemical characteristics. Nanoparticles hold the promise to overcome these problems due to their ability to improve bioavailability, antifungal efficacy and aqueous solubility. Further, drug incorporation into a nanoemulsion (NE) could greatly minimise

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its toxicity. Despite these potential advantages, there are few marketed nanoparticle-based antifungal drug formulations, and thus research into antifungal therapy with nanostructured systems is needed.[13]

Allylic thiocyanates showed moderate-to-high activity against methicillin-resistant *Staphylococcus aureus* (MRSA),[14] *Mycobacterium tuberculosis* (Mtb),[15] human cancer cells[16] and other targets.[17] Therefore, we present the antifungal activity of functionalised allylic thiocyanates derived from the Morita-Baylis-Hillman reaction,[18] as well as the toxicity and anti-inflammatory properties of this collection of sulphur-containing derivatives. The antifungal activity of the most promising compound was also evaluated in NE and in an alternative fungal infection model.

Results

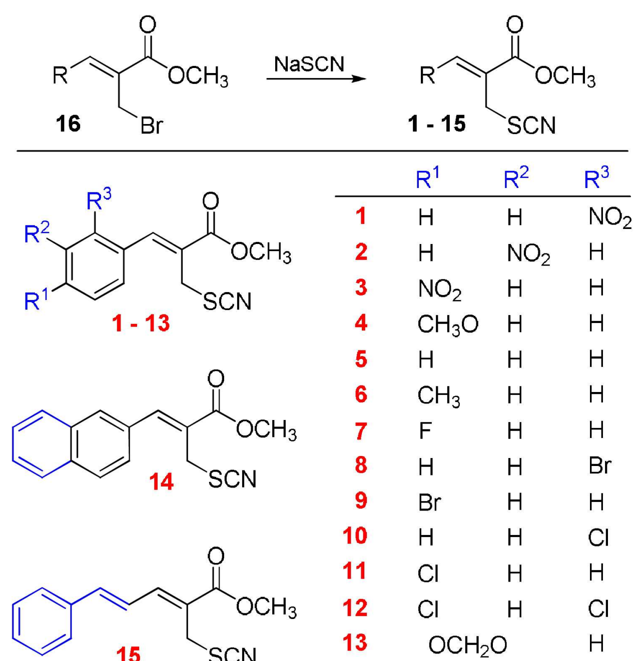
Nanoemulsion

NE was prepared as a white and milky liquid with a macroscopically homogeneous appearance, bluish reflection and a droplet size characteristic of colloidal systems. The pH of the compound **11**-containing NE (**NE-C**, 7.45 ± 0.06) was slightly higher compared to the placebo formulation (prepared without the compound **11** - **NE-WC**, 7.29 ± 0.09). Granulometric analysis of the formulations revealed that the droplets were 190.42 ± 8.64 nm for the NE composed of the active compound **11** - **NE-C** and 225.3 ± 29.67 nm for **NE-WC**. Polydispersion index (PDI) values were 0.120 ± 0.04 and 0.200 ± 0.07 for **NE-C** and **NE-WC**, respectively.

The zeta potential (ZP) values were −8.68 ± 0.31 mV and −7.32 ± 1.41 mV for **NE-C** and **NE-WC**, respectively. ZP increased slightly for **NE-C**, which can be attributed to greater physicochemical stability of the developed colloidal system. In addition, the compound showed high values of total content and encapsulation efficiency in the NE being 96% ± 7.84 and 92%, respectively, which is attributed to the affinity of compound **11** with the oil phase of the NE.

In vitro antifungal susceptibility tests

Eight fungal strains were initially used to evaluate the antifungal activity of allylic thiocyanates **1**–**15** (Scheme 1; Supporting Information, Table S1). Compounds **1**, **4**, **6**, **7**, **10**, **12**, **13** and **15** (Scheme 1) failed to inhibit fungal growth up to the maximum evaluated concentration (50 μg mL^{-1}). Compounds **2**, **3**, **5**, **8**, **9** and **14** (Scheme 1) exhibited fungicide activity against *Candida* spp., with minimal inhibitory concentrations (MICs) of 50 μg mL^{-1}, but were not effective against filamentous fungi. The compound **11** demonstrated a broad fungistatic spectrum against dermatophytes and yeasts, with a MIC of 50 μg mL^{-1} (Supporting Information, Table S1). It is noteworthy that compound **11** inhibited the growth of drug-resistant *T. mentagrophytes*, *C. krusei*, *C. glabrata* and *C. tropicalis* (MICs and breakpoints of commercial antifungal agents are presented in Supporting Information, Table S1).



	R^1	R^2	R^3
1	H	H	NO_2
2	H	NO_2	H
3	NO_2	H	H
4	CH_3O	H	H
5	H	H	H
6	CH_3	H	H
7	F	H	H
8	H	H	Br
9	Br	H	H
10	H	H	Cl
11	Cl	H	H
12	Cl	H	Cl
13	OCH_2O		H

Scheme 1. Chemical structure of allylic thiocyanates **1–15**.

Based on these findings, compound **11** was chosen for the development of a NE.

The MICs of compound **11** in the free form and in a NE for 25 clinical strains of dermatophytes and *Candida* spp. are presented in Table 1. For some species, such as *T. rubrum*, *T. schoenleinii*, *C. parapsilosis* and others, thiocyanate **11** showed a MIC of 25 μg mL^{-1}. With the comparison of the MIC values that inhibited approximately 50% of the clinical strains (MIC_{50}) for the free compound **11** and compound **11**-containing NE (**NE**–**C**) was possible to observe that **NE**–**C** was much more active than the free compound (decrease of MIC values up to 64-fold for dermatophytes and 4-fold for *Candida* spp.). In addition, **NE**–**C** was fungicidal at the MIC concentration for all fungal species analysed. This is an important advantage, since free compound **11** was only fungistatic. **NE-WC** showed no antifungal activity; thus, the other components of the NE do not exert antifungal activity.

In vivo antifungal efficacy in Infected Egg Test-Chorioallantoic Membrane (IET-CAM)

For dermatophytes, infected CAM treated with compound **11** showed eggs without microbial growth in which the embryos were still alive at the time of incubation (Supporting Information, Table S2). We initially counted 1 x 10^2 – 1 x 10^3 conidia mL^{-1}. After the incubation period, the eggs treated with compound **11** showed the absence of fungal growth (Supporting Information, Figure S1). Thus, compound **11** presented 100% efficiency in *in vivo* antifungal activity, considering that the infection did not develop and no embryos died after treatment with the compound. While untreated eggs scored

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Table 1. MIC/MFCs (μg mL^{-1}) of free compound **11** and nanoemulsion containing compound **11** (**NE-C**).

Fungal strains Dermatophytes (n = 15)	Compound **11**	**NE-C**
Microsporum canis (MCA 01)	50/ > 50	1.56/1.56
Microsporum canis (MCA 33)	25/ > 25	0.78/0.78
Microsporum canis (MCA 38)	50/ > 50	0.78/0.78
Microsporum gypseum (MGY5 HCPA)	25/ > 25	1.56/1.56
Microsporum gypseum (MGY 42)	50/ > 50	1.56/1.56
Microsporum gypseum (MGY 50)	50/ > 50	1.56/1.56
Microsporum gypseum (MGY 58)	50/ > 50	1.56/1.56
Trichophyton mentagrophytes (TME 16*)	50/ > 50	0.78/0.78
Trichophyton mentagrophytes (TME 40)	25/ > 25	0.78/0.78
Trichophyton mentagrophytes (TME)	50/ > 50	1.56/1.56
Trichophyton rubrum (TRU 2 HCPA)	25/ > 25	0.78/0.78
Trichophyton rubrum (TRU 3 HCPA)	25/ > 25	0.78/0.78
Trichophyton rubrum (TRU 45)	50/ > 50	0.78/0.78
Trichophyton rubrum (TRU 48)	50/ > 50	1.56/1.56
Trichophyton schoenleinii (TSHO 3 HCPA)	25/ > 25	0.78/0.78
MIC50	50	0.78
MIC range	25 – 50	0.78 – 1.56
Yeasts (n = 10)		
Candida albicans (CA ATCC 18804)	50/ > 50	12.5/12.5
Candida albicans (CA 01)	25/ > 25	25/25
Candida krusei (CK 02)	50/ > 50	50/50
Candida krusei (CK 03)	25/ > 25	12.5/12.5
Candida glabrata (CG 05)	50/ > 50	50/50
Candida glabrata (CG 09)	50/ > 50	50/50
Candida tropicalis (CT ATCC 750)	25/ > 25	12.5/12.5
Candida tropicalis (CT 72 A*)	50/ > 50	50/50
Candida parapsilosis (CP 06)	50/ > 50	12.5/12.5
Candida parapsilosis (CP 07)	25/ > 25	12.5/12.5
MIC_{50}	50	12.5
MIC range	25 – 50	12.5 – 50

*Multidrug-resistant and resistant fungal isolates; MIC, minimal inhibitory concentration; MFC, minimal fungicidal concentration; MIC_{50}, minimal inhibitory concentration that inhibits approximately 50% of the clinical strains analyzed; MIC range, minimum and maximum limits of MIC values; n, number of clinical strains.

between 1 x 10^2 – 1 x 10^5 conidia mL^{-1} (measured in triplicate), all eggs showed fungal growth, and all embryos died.

For the examined yeast strains (Supporting Information, Table S2), fungal growth occurred on some embryos (1 x 10^4 – 1 x 10^5 colony forming units [CFU] mL^{-1}) and consequently led to death. For all clinical *Candida* strains analysed, only one out of the 3 eggs showed fungal growth after treatment with compound **11**; this embryo subsequently died. Thus, compound **11** was 66.6% effective in the yeast infection model (Supporting Information, Table S2).

Embryonic death was evaluated both by egg transluminescence and embryo heartbeat cessation. After treatment with compound **11** at a concentration 4 times higher than the MIC (200 μg mL^{-1}), we observed perfectly formed vessels just below the chorioallantoic membrane (Supporting Information, Figure S1A), while these vessels were absent in untreated controls (Supporting Information, Figure S1B). This finding suggested overall disrupted embryonic development and consequent non-viability.

Antichemotactic assay

Antichemotactic activity was expressed as a percentage of neutrophil migration inhibition relative to controls. Compound **11**, for all tested concentrations, significantly inhibited leucocyte migration. Lipopolysaccharide from *Escherichia coli* (LPS; Table 2) was used as chemoattractant. Complete leucocyte migration inhibition occurred at the maximum evaluated concentration (5 μg mL^{-1}), while the positive control, indomethacin, inhibited approximately 60% of migration at the same concentration. Compound **11** demonstrated the potential for antichemotactic action because at a concentration 10 times lower than the MIC it was able to inhibit leucocyte migration completely.

Table 2. *In vitro* effect of compound **11** and indomethacin compared to negative control.

Samples	Concentration (μg mL^{-1})	Migration (μm)	Migration inhibition (%)
Compound **11**	5	0.0 ± 0.0	100.0*
	1	16.0 ± 2.8	78.0*
	0.1	28.8 ± 8.2	58.6*
	0.01	51.2 ± 8.0	26.2*
	0.001	51.2 ± 4.8	26.2*
Indomethacin			
	5	32.6 ± 7.8	59.7*
	1	34.0 ± 5.7	57.9*
	0.1	40.8 ± 14.5	49.5*
	0.01	95.6 ± 7.7	0
Negative Control	-	80.8 ± 8.4	0

Mean ± standard deviation. *$p < 0.05$ indicates a significant difference compared to negative control (reference chemoattractant - lipopolysaccharide from *Escherichia coli* (LPS)) (ANOVA–Tukey's test).

Antifungal mechanism of action

Sorbitol assay

The MIC of compound **11** against *Candida* spp. and dermatophytes was evaluated in the presence and absence of sorbitol at different times using anidulafungin (AND) as an antifungal control (Supporting Information, Table S3). As expected, the MIC of compound **11** increased after 48, 96 and 168 h due to its fungistatic effect. However, the MICs were the same regardless of being administrated with sorbitol. Meanwhile, the minimal effective concentration (MEC) of AND changed abruptly (more than 8 times) in the presence of sorbitol (Supporting Information, Table S3).

Ergosterol assay

The MIC of compound **11** increased after addition of ergosterol to all strains of *Candida* spp. and dermatophytes. For yeast (*C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata*), MICs increased 4–8-fold after addition of a total of 200 μg mL^{-1} (maximum

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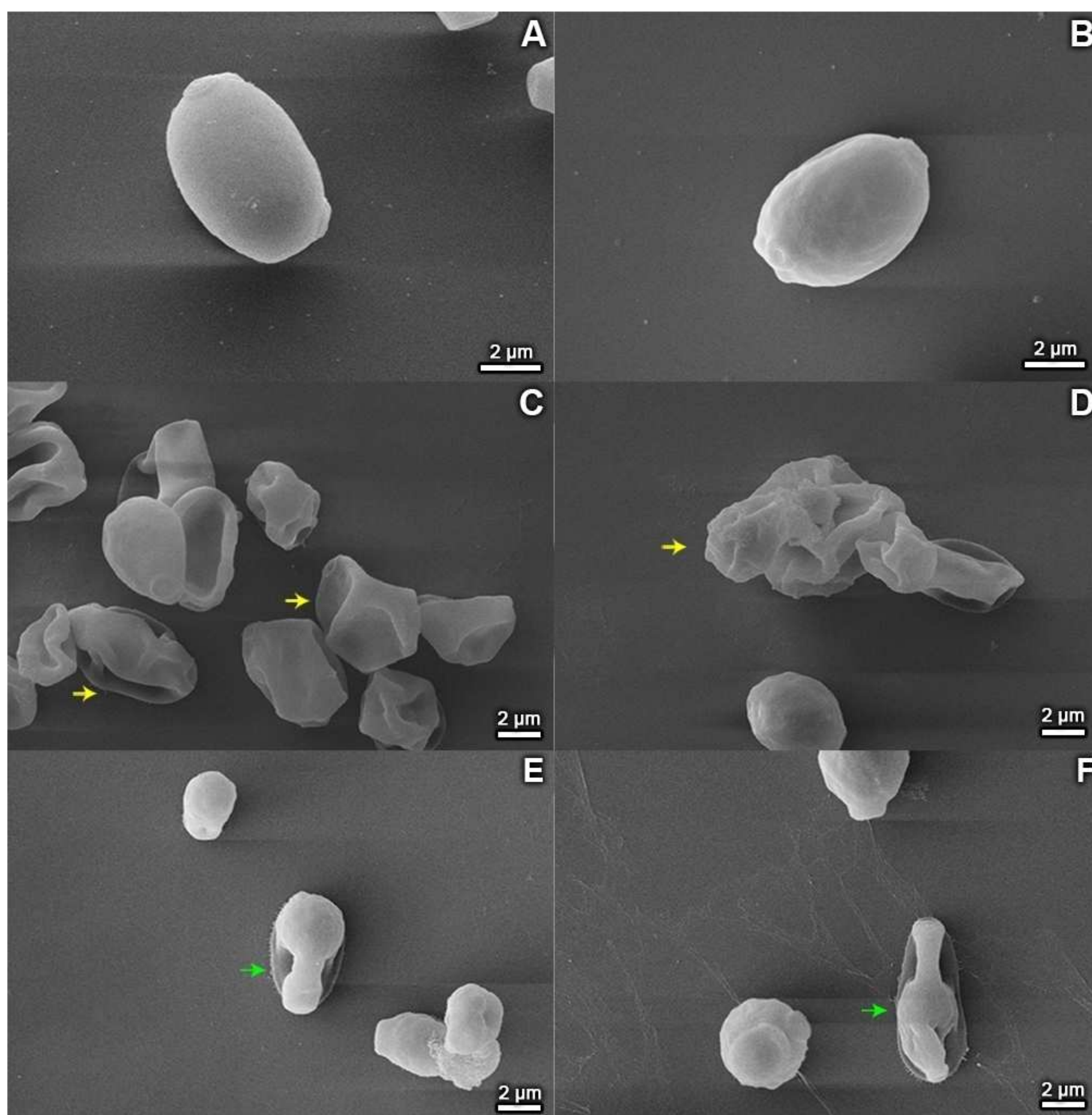

Figure 1. SEM images: (A and B) *C. albicans* ATCC 18804 without treatment (control); (C and D) treated with itraconazole and (E and F) treated with compound **11**.

concentration) of ergosterol during the 5 days of the experiment (Supporting Information, Table S4). As expected, AmB demonstrated an ergosterol-dependent effect. For instance, after addition of 200 μg mL^{-1} of ergosterol, the MIC of amphotericin B (AmB) against *C. tropicalis* increased from 0.5 to >128 μg mL^{-1} (more than 256 times in this case; see Table S4). The same trend was observed for the dermatophytes studied. However, MICs increased more discretely in the case of filamentous fungi (for example, 2-fold for compound **11**; see Table S4).

Scanning electron microscopy (SEM) analysis

C. albicans was treated with either the antifungal itraconazole (ITZ) or the compound **11**. Before addition of the drugs, *C. albicans* cells appeared oval (as expected) without apparent alteration (Figure 1A–B). After treatment with ITZ, changes in cell shape and size were remarkable (Figure 1C–D; yellow arrows point to damaged cells), and damage was also observed from compound **11** treatment (Figure 1E–F; green arrows point to damaged cells). For compound **11**, the fungal cell appeared to rupture, and it was possible to visualise extravasation of intracellular material (Figure 1E; green arrows).

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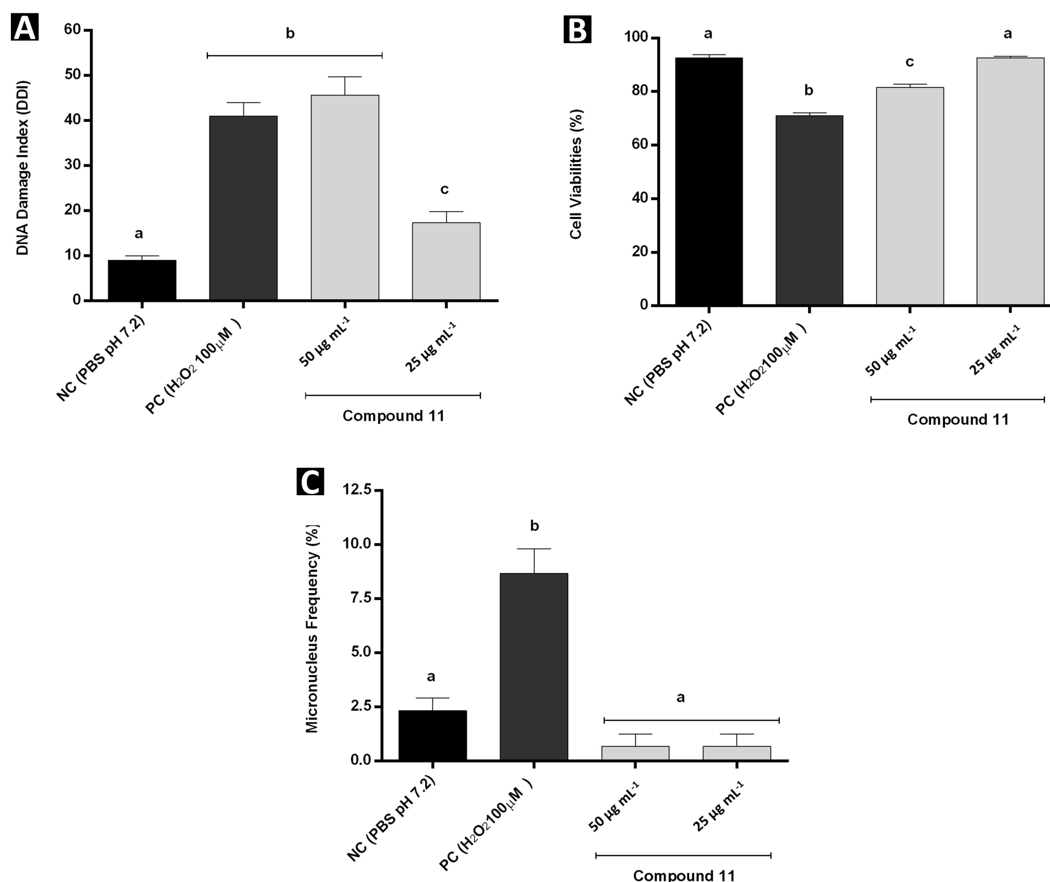

Figure 2. Effect of compound **11** (25 and 50 μg mL^{-1}) in DNA damage (A), cell viability (B) and micronucleus frequency (C). $^{a,b,c}p < 0.05$ indicates significant difference between the controls and the compound **11** (ANOVA followed by Tukey's test).;

Toxicity evaluation

Cytotoxicity, genotoxicity and mutagenicity assays

Compound **11** (50 μg mL^{-1}) caused DNA damage similar to the hydrogen peroxide (H_2O_2; 100 μM) control (Figure 2 - A). However, at 25 μg mL^{-1}, the cell damage was not significant and was comparable to phosphate-buffered saline (PBS, negative control; Figure 2 - A). Approximately 70% and 90% of leucocytes were viable after treatment with compound **11** at 50 and 25 μg mL^{-1}, respectively (Figure 2 - B). Finally, micronucleus was not observed after addition of 25-50 μg mL^{-1} of compound **11** (Figure 2 - C).

Hemolysis

The mean percentage of haemoloysis (± SD) induced by compound **11** at 50 μg mL^{-1} and 100 μg mL^{-1} (concentration 2 times higher than MIC) was 4.20% ± 0.0028 and 4.35% ± 0.0032, respectively. These values are considered very low when compared to water, which causes 100% erythrocyte lysis. PBS did not cause significant haemoloysis. These results corroborated the aforementioned cytotoxicity evaluation in human leucocytes.

Hen's Egg Test-Chorioallantoic Membrane (HET-CAM)

The HET-CAM analysis showed the irritation score (IS) of 3.06 ± 0.50 for the compound **11.** This value classifies the compound as a nonirritant, and suggests it is not allergenic to membranes and thus suitable for future topical use.

Discussion

Previous data showed that compound **11** exhibited moderate-to-good activity against *C. albicans* (12.5 μmol L^{-1}) and *C. tropicalis* (25 μmol L^{-1});[14] these findings corroborate to our results. Furthermore, compounds **8**, **9**, **11** and **14** inhibited MRSA growth,[14] and thus demonstrated a broad spectrum of action against fungi and bacteria. Compound **11** and bromo-substituted analogues **8** and **9** exhibited promising antitubercular activity against replicating and non-replicating forms of Mtb $H_{37}Rv$ (MIC 0.25 μmol L^{-1}) with relatively low toxicity toward VERO cells.[15] Contrarily, 2-chloro-substituted analogues

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10 and **12** were inactive against Mtb (MICs > 128 μmol L^{-1}).[15] The same trend was observed in the current study for adducts **10** and **12**, since both were not active at concentrations up to 50 μg mL^{-1} (Supporting Information, Table S1). Thus, compound **11** is a potential antimicrobial agent that exhibits broad-spectrum activity against MRSA, *Candida* spp., dermatophytes and Mtb. This profile was previously verified for imidazolium salts, with chloro-derived compounds being the most effective *in vitro* antifungal agents when compared to other analogues.[19]

Lipophilicity at the 2- and 3-position of the aromatic ring seems to play a pivotal role for activity (Scheme 1; Supporting Information, Table S1). The best results in this sense were achieved for compounds **8** and **14**, both of which contained more lipophilic groups, such as 2-bromophenyl and 2-naphthyl, respectively. Other less lipophilic analogues, such as the 2-nitro- and 2-chloro-compounds **1** and **10**, were not active up to 50 μg mL^{-1}. The presence of an electron-withdrawing substituent at the aryl group, including nitro (compounds **2** and **3**), bromo (compound **9**) and chloro (compound **11**), seemed to improve activity. The only exception to this trend was the 4-fluoro-substituted (compound **7**) that was also inactive to the endpoint chosen in this study. Meanwhile, thiocyanates containing electron-donating groups, including methoxy (compound **4**) and methyl (compound **6**), were not active up to 50 μg mL^{-1}. Chain elongation was also deleterious to activity (see the cinnamoyl derivative - compound **15**; Supporting Information, Table S1) as well as the simultaneous presence of two substituents at the aromatic ring, such as in compounds **12** and **13**. Hence, our screening of the thiocyanates **1–15** against the present panel of fungi correlated with previous results for these compounds towards Mtb[15] and bacteria,[14] allowed us to select compound **11** as the best lead for further evaluation to develop a new broad spectrum drug and NE to treat microbial skin infections. Therefore, its mechanism of antifungal action, anti-inflammatory capacity and toxicity were determined.

Our NE displayed all characteristics expected to a nanostructure.[20–22] Droplet size and PDI may vary according to the composition of the formulations and the method employed in the preparation.[23] The presence of thiocyanate in the NE reduced the droplet size and the PDI values, and these observations indicate that this compound possess some surface activity that contributes to the formation of smaller and uniform droplets. Additionally, the **NE-C** showed a slightly higher pH value attributed to the weakly basic character of the thiocyano group. Incorporation of compound **11** in the NE (**NE-C**) potentiated its *in vitro* antifungal activity, as shown by the considerable decrease of the MIC values for dermatophytes and yeast (Table 1). AmB is a classic example of an antifungal agent that is associated with improved performance, including the control of drug delivery, lower toxicity and improved effectiveness, when it is administered in nanostructured formulations.[24,25] In addition to the lipophilic nature of the NE, high surface area due to reduced particle size improved drug permeation across biological membranes, and resulted in better drug efficiency, and bioavailability.[13,26]

With regards to *in vivo* testing, an alternative model of fungal infection in embryonated chicken eggs was utilised. Compound **11** reversed the infectious process by dermatophytic clinical strains in 100% of the analyzed eggs. For *Candida* spp., the efficiency of compound **11** was 66.6%. Thus, in addition to broad spectrum *in vitro* action, compound **11** was also effective *in vivo* by greatly reducing the microbial load associated with the infectious process, including for drug-resistant species. It is important to emphasise that our study represents, for the first time, an alternative model of fungal infection in chicken eggs that has been applied for the evaluation of antifungal activity of new small molecules. Embryonic death in the treated eggs may be linked to infection by *Candida* spp. or filamentous fungi strains, but there were also eggs treated with compound **11** that resulted in dead embryos. These results, and the deaths of the embryos in the controls without inoculum, can be explained by manipulation of the eggs, but may also be related to genetic defects or embryonic development; the latter two factors are independent of the experimental procedure. The infection dose of 1 x 10^3 CFU mL^{-1} was considered low and was likely not the determining factor for the embryo inviability.[27]

The Boyden chamber method (antichemotactic assay) was used to evaluate whether compound **11** would inhibit polymorphonuclear neutrophil migration and to analyze the anti-inflammatory properties of the compound. Leucocyte migration to the site of injury is considered one of the first major steps for inflammation.[28] Our results suggested that compound **11** acted in response to an acute inflammatory process (Table 2). Mechanisms that promote inflammation and impair the antifungal immune response are continually discovered. It is known, for example, that *C. albicans* and *Aspergillus fumigatus* colonizations are associated with elevated levels of pro-inflammatory cytokines (IL-17, IL-23 and Th17).[11,29] Fungal colonization, however, does not necessarily imply infection and disease development. The stability of the host–fungus relationship is maintained by a complex balance of pro- and anti-inflammatory intracellular signals.[30,31] Consequently, control of the inflammatory response may represent a strategy to combat fungal infections.[11] Dermatophyte metabolites generally induce inflammation at the site of infection.[32] Occasionally, accentuated inflammatory responses are also associated with increased severity and chronicity of mycoses.[31] Therefore, an antifungal substance with related anti-inflammatory properties will likely more effectively ameliorate a fungal infection.[33] Compound **11** significantly reduced neutrophil migration (part of the acute phase of inflammation) at 0.1 to 5 μg mL^{-1} (10 times lower than the MIC), and these results suggest that this compound would work as a drug to treat fungal infections associated with inflammatory disorders.

Sorbitol exerts osmotic protection on the fungal cell wall by blocking chemicals from acting on this target,[34] and antifungal activity will decrease in the presence of sorbitol if a drug acts on the cell wall. Our results indicated that the antifungal effect of compound **11** was not related to the cell wall, since the MIC values did not vary with the addition of sorbitol.

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Some antifungal drugs act by interacting with ergosterol in the cell membrane; ergosterol is an important target since it is not present in mammalian cells. The addition of ergosterol in growth medium will increase the concentration of this substance outside of the membrane and allow the drug to more easily interact with it. Consequently, if the drug's mechanism of action involves the cell membrane, it would become less active (higher MICs).[35,36] Thus, MICs of the compound **11** against all fungi strains were determined in the presence of exogenous ergosterol. AmB, a commercial antifungal, was used as control drug (Supporting Information, Table S4). MICs of compound **11** increased after addition of ergosterol to all *Candida* spp. and dermatophytes, and these findings suggested a mechanism of action related to complexation with ergosterol in the cell membrane, as similarly observed for AmB. The loss of activity in the presence of exogenous ergosterol was time- and dose-dependent for *Candida* spp. (Supporting Information, Table S4). Since our results indicated that compound **11** acts on the fungal cell membrane, we next evaluated the effect of **11** on the fungal cellular structure by SEM (Figure 1). *C. albicans* was treated with either the antifungal ITZ or the compound **11**. In both cases, treated cells lost their internal contents in a process characteristic of plasmolysis. This observation corroborated the proposed mechanism of action of compound **11** against all *Candida* spp. and dermatophytes strains presented in this study.

Next, cytotoxicity studies demonstrated that the cell damage was directly related to the concentration of compound **11**. The mutagenic effect of **11** evaluated by the micronucleus assay indicated that this compound does not generate mutations at the concentration necessary for *in vitro* antifungal activity (Figure 2). Besides, compound **11** did not cause significant leucocyte death at the evaluated concentrations (Figure 2). While **11** did not cause significant haemoloysis, we observed a genotoxic effect at 50 μg mL^{-1}.

Overall, a future topical formulation that contains the chloro-substituted compound **11** would be safe. This supposition is corroborated by the lack of allergenicity by HET-CAM. The HET-CAM, an alternative to the Draize test, mimics vascular changes in the chorioallantoic membrane as a model for the conjunctival ocular surface and can be a qualitative method of assessing the potential irritancy of chemicals.[37] Besides that, our results demonstrated that embryonated eggs are highly susceptible to yeast and dermatophytes infection via the CAM since these fungi proliferate radially on tissue and blood vessels. The compound **11** was classified as nonirritant by the HET-CAM assay . This result is a good indication of general low membrane toxicity.

Conclusions

Fifteen allylic thiocyanates were screened against a panel of *Candida* spp. and filamentous fungi. Six compounds exhibited fungicide activity against *Candida* spp. at 50 μg mL^{-1}. The 4-chlorophenyl-substituted compound **11** demonstrated a fungistatic effect (50 μg mL^{-1}) against the entire fungal panel, and exhibited anti-inflammatory capability by reducing neutrophil migration. These results designate compound **11** as a possible complement to conventional antifungal therapy with the advantage of an anti-inflammatory effect, which can accelerate the relief of symptoms, facilitate healing and prevent infection dissemination. The incorporation of the compound **11** in a NE greatly potentiated the *in vitro* antifungal activity as denoted by reduced MIC values ($MIC_{50} = 0.78$ μg mL^{-1} for dermathophytes and 12.5 μg mL^{-1} for *Candida* spp.). In the *in vivo* assay, compound **11** completely eliminated the dermatophytosis of infected egg chorioallantoic membrane. The mechanism of action of compound **11** was not related to the fungal cell wall since MICs were not altered in the presence of sorbitol. However, compound **11** formed an ergosterol complex similar to that observed for AmB, and this complex is possibly related to its broad-spectrum activity. SEM images suggested cell damage through plasmolysis and modifications of the regular yeast cell shape. Compound **11** also caused dose-dependent DNA damage in human leucocytes. Micronucleus did not occur after treatment with compound **11** at 25-50 μg mL^{-1}, and this finding indicated that the compound does not induce mutations in human leucocytes at concentrations that produce *in vitro* antifungal activity. In addition, 70% and 90% of leucocytes became viable after treatment with compound **11** at 50 μg mL^{-1} and 25 μg mL^{-1}, respectively; the compound did not cause haemolysis. The results of HET-CAM classified this compound as nonirritant. In addition to its antifungal, antibacterial[14] and antituberculosis activities,[15] compound **11** can be easily prepared from inexpensive and readily available chemicals. NE that use compound **11** as the active component can be a future alternative or a complement to conventional treatments for cutaneous mycoses caused by yeast and filamentous fungal pathogens.

Supporting Information Summary

Details of the experimental method can be found in the electronic Supporting Information, as well as complementary results such as complete tables of minimal inhibitory concentration and figures that support the main results reported in this article.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Allylic thiocyanates · Antifungal activity · Nanoemulsion · Toxicity

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Supporting Information

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Nanoemulsion Improves the Antifungal Activity of Allylic Thiocyanates against Yeasts and Filamentous Pathogenic Fungi

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SUPPORTING INFORMATION

Experimental

Chemical Synthesis

Fifteen allylic thiocyanates **1**–**15** (Scheme 1) were readily synthesized from the corresponding allylic bromide **16** (Scheme 1) according to previously described methods.[1–4] The typical procedure for the synthesis of allylic thiocyanates **1**–**15**, consists of a stirred solution of allylic bromide **16** (1.0 mmol) in 4.0 mL of acetone/H_2O (3:1 v/v) at 25 °C was added 2.0 mmol of NaSCN. After stirring for 1 h, the final mixture was diluted with CH_2Cl_2 and washed with H_2O and brine. The organic extract was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by chromatography (hexane/ethyl acetate 9:1) to give the corresponding (*Z*)-2-(thiocyanomethyl)alkenoates. Spectral and analytical data for the novel compound **2**: 2 Methyl (*Z*)-3-(3-nitrophenyl)-2-(thiocyanomethyl)-2-propenoate. Yield 98%; white solid, mp 71.0-72.0 °C. IR (KBr): ν_{max}/cm^{-1} 3085, 2952, 2155, 1716, 1532, 1351, 1270. ^1H NMR (400 MHz, $CDCl_3$): δ 3.90 (s, 3H), 4.04 (s, 2H), 7.67 (t, *J* = 8.0 Hz, 1H), 7.75-7.78 (m, 1H), 8.00 (s, 1H), 8.22-8.27 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 30.8, 53.2, 111.7, 124.1, 124.5, 129.1, 130.5, 135.0, 135.5, 141.9, 148.7, 165.8. Anal. Calcd for $C_{12}H_{10}N_2O_4S$ (%): C, 51.79; H, 3.62; N, 10.07. Found: C, 51.78; H, 3.55; N, 9.98.

Each compound **1**–**15** was solved in DMSO and diluted using sterile ultrapure water to give solutions having DMSO in concentrations lower than 1% for subsequent investigation of antifungal/antichemotactic activities and toxicity determinations.

Preparation and physicochemical characterization of nanoemulsion (NE)

The NE were prepared in triplicate by the spontaneous emulsification solvent diffusion method, where an organic phase preheated to 40 °C, composed of the active compound **11** (**NE-C**, 5 mg), the oil (medium chain triglycerides – MCT, 0.15 g), the SPAN® 80 (0.077 g) and the solvent (ethanol, 27 mL), was injected under magnetic stirring into an aqueous phase (distilled water, 53 mL) containing Tween® 80 (0.077 g). The emulsion formed was kept under magnetic stirring for 10 min and then the organic solvent and part of the aqueous solvent were removed on a rotary evaporator to a final volume of 10 mL. For comparison purposes, a NE was prepared without the compound **11** (**NE-WC**).

The NEs were submitted to a physicochemical characterization, where all the parameters were evaluated in triplicate. The pH of the NEs was determined directly on the samples by the use of a potentiometer. The NEs diameter and polydispersion index (PDI) evaluation was performed by photon correlation spectroscopy, after adequate dilution of an aliquot of the samples in ultrapure water (1:500) (Zetasizer Nanoseries, Malvern Instruments, UK). The zeta potential (ZP) values were determined by micro electrophoresis after dilution of the formulations into 10 mM NaCl solution.

The compound **11** content in the formulations as well as the encapsulation efficiency was evaluated by high performance liquid chromatography (HPLC). For this, an aliquot of the samples was diluted in 10 mL of methanol and subsequently sonicated for 10 min to compound extraction. After, samples were filtered through a 0.45 µm membrane and injected into the HPLC system. Chromatographic instruments and conditions were the following: LC-10A HPLC system (Shimadzu, Japan) equipped with a LC-20AT pump, an UV-VIS SPD-M20A detector, a CBM-20A system controller and a SIL-20A HT valve sample automatic injector. Separation was achieved at room temperature using an Inertsil ODS-3 C_{18} Gel Sciences column (150 mm × 4.6 mm, 5 µm) coupled with a C_{18} guard column. The isocratic mobile phase consisted of methanol and water (80:20, v/v) at 1.2 mL min^{-1} flow rate. The compound was detected at 284 nm with a retention time of about 4.03 min. The analytical methodology was previously validated. The method was found to be linear (r = 0.995) at the concentration range of 2.5–12.5 µg mL^{-1} and specific.

The encapsulation efficiency was determined by ultrafiltration/centrifugation technique. An aliquot of the samples was placed in a 10.000 MW centrifugal device (Amicon®Ultra, Millipore) and free compound was

separated at 2200 × g for 10 min. The ultrafiltrate was analyzed by HPLC method. The encapsulation efficiency (%) was calculated from the difference between the total and free drug concentrations. The results of this analysis were expressed as averages followed by standard deviations (SD).

In vitro **antifungal susceptibility test**

Fungal strains

Yeast species of the genus *Candida* (*C. albicans* - CA ATCC 18804, *C. krusei* - CK 02*, *C. glabrata* - CG 09*, and *C. tropicalis* - CT 72A*) and dermatophytic filamentous fungi (*Microsporum canis* - MCA 01, *Microsporum gypseum* - MGY 42, *Trichophyton mentagrophytes* - TME 16*, and *Trichophyton rubrum* - TRU 45) were selected for the screening of antifungal activity. Subsequently, for the comparison of the free compound and incorporated in the nanoformulation (**NE-C**), more yeasts and dermatophytes strains were included in this study. All fungal species are deposited in the Mycology Collection of the research group in Applied Mycology, Faculty of Pharmacy (Universidade Federal do Rio Grande do Sul, Brazil). For *Candida* spp. the resistance (*) was defined based on Kuriyama et al. (2005)[5] and CLSI breakpoints,[6,7] considering in this case the clinical isolates CK 02* resistant to: itraconazole (MIC = 1 µg mL^{-1}) and fluconazole (MIC ≥ 64 µg mL^{-1}), GC09* itraconazole (MIC > 4 µg mL^{-1}) and miconazole (MIC = 8 µg mL^{-1}), and CT 72A* resistant to itraconazole (MIC = 1 µg mL^{-1}), miconazole (MIC > 8 µg mL^{-1}), and voriconazole (MIC = 2 µg mL^{-1}). As for dermatophytes, the resistance (in the sense of reduced susceptibility) was established according to the increase in minimal inhibitory concentration (MIC) values for some clinical strains in relation to the majority, considering the following resistance threshold concentrations: terbinafine - MIC ≥ 1 µg mL^{-1}, griseofulvin - MIC ≥ 4 µg mL^{-1}, and ketoconazole - MIC ≥ 8 µg mL^{-1}. Consequently, the clinical isolate TME 16* was considered multidrug-resistant by the considerable elevation of MICs of three antifungal agents of different classes (MIC terbinafine = 4 µg mL^{-1}, MIC griseofulvin > 32 µg mL^{-1} and MIC ketoconazole = 16 µg mL^{-1}).

Antifungal agents

Terbinafine (TBF), amphotericin B (AmB) and anidulafungin (AND), purity ≥ 97%, were supplied by Cristalia (Sao Paulo, Brazil), griseofulvin (GSF), purity ≥ 97%, was acquired from Wallace Pharmaceuticals (Mumbai, India), ketoconazole (KTZ), purity ≥ 96%, was obtained from All Chemistry (Sao Paulo, Brazil), fluconazole (FCZ) purity ≥ 98% was obtained from Sigma-Aldrich (Sao Paulo, Brazil), itraconazole (ITZ) purity ≥ 97%, was supplied by Cassará laboratory (Dist. PHARMOS; Buenos Aires, Argentina), miconazole (MCZ) purity ≥ 97%, was supplied by Valdequimica Chemical Products (Sao Paulo, Brazil), and voriconazole (VRZ); purity ≥ 98%, was supplied by Pfizer (Sao Paulo, Brazil). The preparation of stock and work solutions followed the recommendations of the CLSI.[8] The commercial antifungals were used as reference substances for comparison with the synthetic compounds in the tests conducted.

Determination of MIC and minimal fungicidal concentration (MFC)

The series of synthetic compounds and the NE containing the compound **11** were evaluated for MIC determination, through the broth microdilution technique.[6–8] The inocula of yeasts (0.5x10^3 to 2.5x10^3 CFU mL^{-1}) and dermatophytes (1.0x10^3 to 3.0x10^3 CFU mL^{-1}) were prepared from cultures grown on sabouraud dextrose agar (SDA; Kasvi, Brazil) and potato dextrose agar (PDA; Neogen, USA), respectively. [6–8] Posteriorly, aliquots of each serial microdilution (corresponding to MIC, 2xMIC, and 4xMIC) were spread on SDA (*Candida*) and PDA (dermatophytes), incubated at 35 °C,[6–8] and analyzed to determine the MFC, which was defined as the lowest concentration that yielded up to three colonies.[9]

In vivo **antifungal efficacy in Infected Egg Test-Chorioallantoic Membrane (IET-CAM)**

Fresh and fertile white eggs were kept under optimized incubation conditions (38−39 °C, 55−60% humidity, 12 days). On the 4th day, the CAM of eggs were infected with 0.1 mL inoculum 4x/day until the occurrence of the infectious process was confirmed by the visualization of colonies onto the CAM. The preparation of the yeast ($4x10^3$ CFU mL^{-1}) and filamentous fungi ($1.0x10^3$ CFU mL^{-1}) inocula followed the CLSI.[6–8] On the 8th day of incubation, the treatment was started. An aliquot of 0.1 mL of compound **11** (200 µg mL^{-1}, 0.5% DMSO solution) and the negative control (0.9% NaCl) was added onto the CAM. On the 12th day, eggs were reopened. 0.1 mL of the embryonic contents were removed, spread on sabouraud agar plates, incubated and the viability of the embryo verified.[10] Subsequently, counting of colony forming units was performed.[10] The study was submitted and approved by the Committee on Ethics in the Use of Animals (CEUA nº 4/2016 - Instituto Federal Catarinense, Concordia, Brazil). The experiment was carried out in triplicate.

Antichemotactic assay

The evaluation of antichemotactic activity was performed according to the method of the modified Boyden chamber as described by Suyenaga et al. (2011).[11] Prior to assay, neutrophils were treated with the compound **11** dissolved in Hank's balanced salt solution (HBSS, pH 7.4) in concentrations of 0.001 to 10 µg mL^{-1}, at 37 °C for 30 min. As negative control was used a neutrophils solution with no addition of antichemotactic agent. Indomethacin was used as positive control. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Rio Grande do Sul (Permission no. 32226, approved on April 24, 2017).

Investigation of antifungal mechanism of action

Sorbitol assay

The sorbitol solution (0.8 M, Sigma-Aldrich) was prepared and diluted in the culture medium (RPMI 1640; Sigma-Aldrich). Then, microplates were incubated at 35 °C for 168 h. The MIC was visually determined in the assay to compound **11**, in the presence and absence of sorbitol.[12] The minimal effective concentration (MEC), which is the lowest concentration of antifungal agent that leads to the growth of small, round and compact hyphal forms, was determined in triplicate only for the antifungal AND (drug control).[6–8]

Ergosterol assay

The susceptibility test was performed in triplicate according to the CLSI[6–8], in the presence and absence of the exogenous ergosterol[13] (Sigma-Aldrich), with compound **11** and using amphotericin B (AmB) as drug control.[13] The microplates were incubated (35 °C, 168 h) and MICs were determined visually in the presence and absence of exogenous ergosterol, in different concentrations and times.[13]

Scanning electron microscopy (SEM) analysis

After the incubation period defined by the susceptibility test, wells containing the coverslips were washed three times with phosphate-buffered saline (PBS). After washing, adhered cells received 500 µL of glutaraldehyde (2.5%, type 1, Sigma-Aldrich), diluted with sodium cacodylate (0.1 mol L^{-1}, pH 7.2, Sigma-Aldrich), and kept for 1 h at room temperature. Then, the wells were washed three times with sodium cacodylate (0.1 mol L^{-1}, pH 7.2) containing sucrose (0.2 mol L^{-1}) and $MgCl_2$ (2 mmol L^{-1}). Adhered cells were dehydrated in a series of freshly prepared solutions of ethanol (30, 50, and 70%, for 5 min/step, 95% and 2x100%, for 10 min/step). Samples were subjected to critical point drying (EM CPD 300, Leica), mounted on metallic stubs, sputter-coated with a 15–20 nm gold-palladium layer, and visualized in a scanning electron microscope (Carl Zeiss EVO® MA10, Germany) operating at 10 kV. The images were performed with a strain of *C. albicans* (CA ATCC 18804) without treatment (control) and treated with compound **11** (25 µg mL^{-1} - subinhibitory concentration) and ITZ (1 µg mL^{-1}, drug control).

Toxicity evaluation

Cell culture, cytotoxicity, genotoxicity and mutagenicity

Cell cultures of human leukocytes were prepared using venous blood collected by venipuncture from a male volunteer (protocol #23.081.005770/009-38). Aliquots (1 mL) of whole blood were immediately transferred to 10 mL of RPMI 1640 medium supplemented with 1% phytohemagglutinin, 10% fetal bovine serum and 1% streptomycin/penicillin.[14] Subsequently, cell culture treatments were performed with compound **11** (25 µg mL^{-1} and 50 µg mL^{-1}, in 0.5% DMSO), hydrogen peroxide solution (H_2O_2, 100 µmol L^{-1}, positive control), and PBS (negative control). Cell cultures were incubated (CO_2 incubator for cell culture, 5% CO_2, Model MCO-19AIC, Sanyo) at 37 °C for 72 h.[15] Cytotoxic, genotoxic, and mutagenic parameters were established, in triplicate. Cell viability was assessed with 0.2% trypan blue (Sigma-Aldrich), according to Burow et al. (1998).[15] Genotoxicity was performed by the comet assay. Cells were classified according varying from 0 (no visible damage) to 4 (maximum damage) to provide a unique damage index (ID) from 0 to 400.[16] The assessment of mutagenicity was carried out by Panótico Rápido® (Laborclin), wherein all particles within the cells separated from the nucleus are accounted for as micronuclei (MN).[14,17]

Hemolysis assay

The hemolysis assay was performed using rabbit blood. After collection, the blood was mixed with the anticoagulant K2-EDTA. Rabbit erythrocytes were harvested by centrifugation for 5 min at 400 rpm and washed three times in PBS. A suspension of the 1% erythrocytes was prepared in PBS. The compound **11** solution was prepared and incubated with the erythrocyte suspension for 15 min h at 37°C. After incubation, the cells were spun down by centrifugation, the supernatant was transferred to a 96-well plate and the absorbance (650 nm) measured using a microplate spectrophotometer (BioTek Instruments). Two controls were used in this assay: (i) PBS was used as a negative control (0% hemolysis), and (ii) water was used as positive control (100% hemolysis).

Hen's Egg Test-Chorioallantoic Membrane (HET-CAM)

Fresh and fertile white eggs (Lohmann selected Leghorn, LSL) were kept under optimized incubation conditions (38–39 °C, 55–60% humidity, 10 days). On the 10th day, the eggshell, around the airspace, was removed with a rotary tool (Dremel, WI). Subsequently, 0.3 mL of compound **11** (200 µg mL^{-1}, 0.5% DMSO solution) and controls (negative control: 0.9% NaCl; positive control: 0.1 mol L^{-1} NaOH) were added to the CAM of the eggs.[18] The irritant effect was observed at three times: 30 sec, 2 min and 5 min after application of compound **11** and controls. The result of the irritation score (IS) was calculated according to the [Eq. (1)][18] and presents a maximum value of 21. The eggs were analyzed in relation to the appearance of hemorrhaging, lysis, and coagulation. Classification criterion used: 0 to 4.9 nonirritant (or practically no irritation); 5.0 to 21 irritant (moderate to severe or extreme irritation). [18] The assay was performed in triplicate.

Equation 1. Formula for determination of irritation score (IS)

$$IS = \left(\left(\frac{(301 - \text{hemorrhage time})}{300}\right) x5\right) + \left(\left(\frac{(301 - \text{lysis time})}{300}\right) x7\right) + \left(\left(\frac{(301 - \text{coagulation time})}{300}\right) x9\right)$$

Statistical analysis

Differences between the control and treatments were statistically analyzed by ANOVA followed by Tukey's test (p <0.05 was considered statistically significant). Data analysis was performed using the GraphPad Prism 5.0 software and expressed as mean ± SD.

Results

Tables

Table S1. MIC/MFCs (μg mL^{-1}) for allylic thiocyanates **1**–**15.**

Table S2. Number of eggs with and without fungal growth and number of eggs with live and dead embryos after treatment with compound **11** and controls.

Table S3. MICs (μg mL^{-1}) for the compound **11** and MECs (μg mL^{-1}) for AND, in the presence and absence of sorbitol.

Table S4. MICs (μg mL^{-1}) for the compound **11** and AmB, in the presence and absence of ergosterol.

Figures

Figure S1. Macroscopic changes in infected embryonated eggs; (A) Treatment with compound **11** (200 μg mL^{-1}) (B) Control, without treatment.

Table S1. MIC/MFCs (µg mL^{-1}) for allylic thiocyanates **1–15.**

Compounds and antifungal drugs	Dermatophytes				*Candida* spp.			
	MCA 01	**MGY 42**	**TME 16***	**TRU 45**	**CA ATCC 18804**	**CK 02***	**CG 09***	**CT 72A***
1	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50
2	>50/>50	>50/>50	>50/>50	>50/>50	50/50	50/50	50/50	50/50
3	>50/>50	>50/>50	>50/>50	>50/>50	50/50	50/50	50/50	50/50
4	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50
5	>50/>50	>50/>50	>50/>50	>50/>50	50/50	50/50	50/50	50/50
6	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50
7	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50
8	>50/>50	>50/>50	>50/>50	>50/>50	50/50	50/50	50/50	50/50
9	>50/>50	>50/>50	>50/>50	>50/>50	50/50	50/50	50/50	50/50
10	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50
11	50/>200	50/>200	50/>200	50/>200	50/>200	50/>200	50/>200	50/>200
12	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50
13	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50
14	>50/>50	>50/>50	>50/>50	>50/>50	50/50	50/50	50/50	50/50
15	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50
TBF	0.03 (S)	0.03 (S)	4 (R*)	0.06 (S)	-	-	-	-
GSF	1 (S)	1 (S)	>32 (R*)	1 (S)	-	-	-	-
KTZ	0.5 (S)	1 (S)	16 (R*)	1 (S)	0.25 (S)	1 (S)	0.5 (S)	1 (S)
FCZ	-	-	-	-	1 (S)	≥ 64 (R)	0.25 (DDS)	2 (S)
ITZ	-	-	-	-	-	1 (R)	>4 (R)	1 (R)
MCZ	-	-	-	-	-	0.5 (S)	>8 (R)	>8 (R)
VRZ	-	-	-	-	-	-	-	2 (R)

*Multidrug-resistant and resistant fungal isolates; MIC, minimal inhibitory concentration; MFC, minimal fungicidal concentration; MCA, *Microsporum canis*; MGY, *Microsporum gypseum*; TME, *Trichophyton mentagrophytes*; TRU, *Trichophyton rubrum*; CA, *Candida albicans*; CK, *Candida krusei*; CG, *Candida glabrata*; CT, *Candida tropicalis*. TBF, terbinafine; GSF, griseofulvin; KTZ, ketoconazole; FCZ, fluconazole; ITZ, itraconazole; MCZ, miconazole; VRZ, voriconazole; R, resistance; R*, resistance in the sense of reduced susceptibility compared to other strains; S, susceptible; IR, intermediary resistance; DDS, dose-dependent susceptibility.[6–8,19]

Table S2. Number of eggs with and without fungal growth and number of eggs with live and dead embryos after treatment with compound **11** and controls.

Fungi	**Compound 11**				Efficiency (%)	**Control 1**				**Control 2**				**Control 3**			
	Eggs		Embryos			Eggs		Embryos		Eggs		Embryos		Eggs		Embryos	
	With microbial growth	Without microbial growth	Deads	Lives		With microbial growth	Without microbial growth	Deads	Lives	With microbial growth	Without microbial growth	Deads	Lives	With microbial growth	Without microbial growth	Deads	Lives
MCA 01	0	3	0	3	100	3	0	3	0	2	1	2	1	1	2	1	2
MGY 42	0	3	0	3	100	3	0	3	0	3	0	3	0	2	1	2	1
TME 16*	0	3	0	3	100	3	0	3	0	3	0	3	0	1	2	1	2
TRU 45	0	3	0	3	100	3	0	3	0	3	0	1	2	3	0	3	0
CA ATCC 18804	1	2	1	2	66.6	3	0	2	1	1	2	0	3	0	3	0	3
CK 02*	1	2	1	2	66.6	3	0	2	1	1	2	0	3	0	3	0	3
CG 09*	1	2	1	2	66.6	3	0	3	0	3	0	1	2	2	1	0	3
CT 72A*	1	2	1	2	66.6	3	0	2	1	3	0	1	2	1	2	0	3

*Multidrug-resistant and resistant fungal isolates; MCA, *Microsporum canis*; MGY, *Microsporum gypseum*; TME, *Trichophyton mentagrophytes*; TRU, *Trichophyton rubrum*; CA, *Candida albicans*; CK, *Candida krusei*; CG, *Candida glabrata*; CT, *Candida tropicalis*. **Control 1** consists of chorioallantoic membrane of egg inoculated with the clinical strains; **Control 2** normal eggs without inoculation and without treatment; and **Control 3** normal eggs without inoculation and with compound **11**.

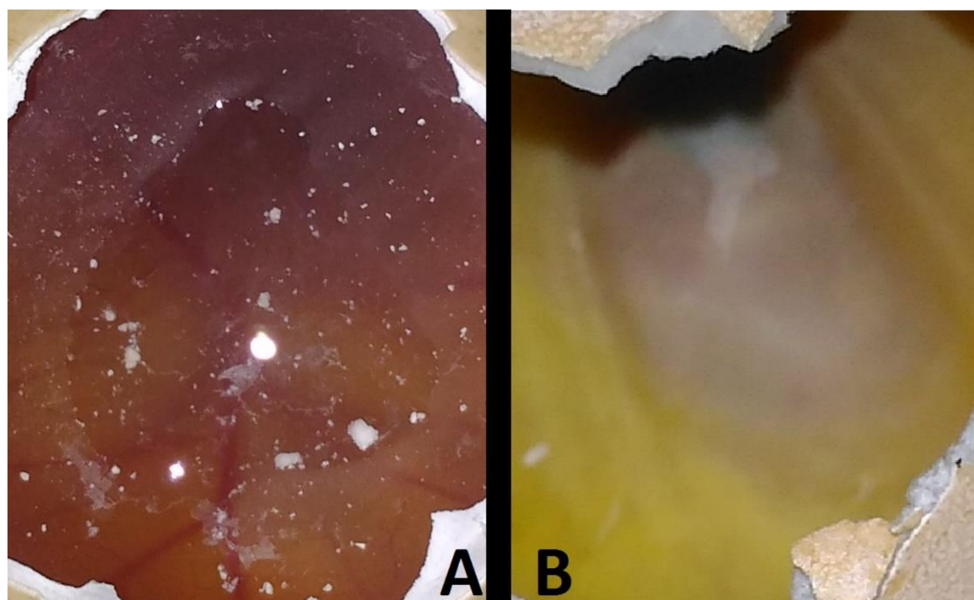

Figure S1. Macroscopic changes in infected embryonated eggs; (A) Treatment with compound **11** (200 μg mL^{-1}) (B) Control, without treatment.

Table S3. MICs (μg mL^{-1}) for the compound **11** and MECs (μg mL^{-1}) for AND, in the presence and absence of sorbitol.

Time	**48 h**				**96 h**				**168 h**			
Dermatophytes and *Candida* spp.	**Compound 11**		**AND**		**Compound 11**		**AND**		**Compound 11**		**AND**	
	AS	**PS**	**AS**	**PS**	**AS**	**PS**	**AS**	**PS**	**AS**	**PS**	**AS**	**PS**
MCA 01	-	-	-	-	50	50	32	128	>800	>800	64	>256
MGY 42	-	-	-	-	50	50	32	128	>800	>800	64	>256
TME 16*	-	-	-	-	50	50	32	128	>800	>800	64	>256
TRU 45	-	-	-	-	50	50	32	128	>800	>800	64	>256
CA ATCC 18804	50	50	0.12	32	100	100	0.12	>64	>800	>800	0.12	>64
CK 02*	50	50	0.12	32	100	100	0.12	>64	>800	>800	0.12	>64
CG 09*	50	50	0.12	32	100	100	0.12	>64	>800	>800	0.12	>64
CT 72A*	50	50	0.12	32	100	100	0.12	>64	>800	>800	0.12	>64

*Multidrug-resistant and resistant fungal isolates. Abbreviations: MIC, minimal inhibitory concentration; MEC, minimal effective concentration; AND, anidulafungin; AS, absence of sorbitol; PS, presence of sorbitol; MCA, *Microsporum canis*; MGY, *Microsporum gypseum*; TME, *Trichophyton mentagrophytes*; TRU, *Trichophyton rubrum*; CA, *Candida albicans*; CK, *Candida krusei*; CG, *Candida glabrata*; CT, *Candida tropicalis*.

Table S4. MICs (μg mL^{-1}) for the compound **11** and AmB, in the presence and absence of ergosterol.

Fungi strains	**Reading 1** (μg mL^{-1})					**Reading 2** (μg mL^{-1})				
Compound 11	**MIC^{1}**	**MIC^{2}**	**MIC^{3}**	**MIC^{4}**	**MIC^{5}**	**MIC^{1}**	**MIC^{2}**	**MIC^{3}**	**MIC^{4}**	**MIC^{5}**
MCA 01	50	50	50	50	50	50	50	50	50	100
MGY 42	50	50	50	50	50	50	50	50	50	100
TME 16*	50	50	50	50	50	50	50	50	50	100
TRU 45	50	50	50	50	50	50	50	50	50	100
CA ATCC 18804	50	50	100	100	200	50	100	100	100	200
CK 02*	50	50	50	100	100	50	50	100	200	200
CG 09*	50	50	50	200	200	50	50	100	400	400
CT 72A*	50	50	100	200	400	50	100	100	400	400
AmB	**MIC^{1}**	**MIC^{2}**	**MIC^{3}**	**MIC^{4}**	**MIC^{5}**	**MIC^{1}**	**MIC^{2}**	**MIC^{3}**	**MIC^{4}**	**MIC^{5}**
MCA 01	0.5	2	4	4	8	0.5	2	4	4	8
MGY 42	4	4	4	8	16	4	4	4	8	16
TME 16*	2	8	8	8	16	2	8	16	16	32
TRU 45	2	2	4	4	8	2	8	16	16	32
CA ATCC 18804	1	2	4	16	16	1	2	16	128	128
CK 02*	1	2	4	16	16	1	2	128	128	128
CG 09*	2	2	4	8	16	2	2	128	128	128
CT 72A*	0.5	2	4	32	128	0.5	2	>128	> 128	> 128

*Multidrug-resistant and resistant fungal isolates. AmB = Amphotericin B; CA = *C. albicans* (CA ATCC 18804); CT = *C. tropicalis* (CT 72A*); CK= *C. krusei* (CK 02); CG= *C. glabrata* (CG 09); MCA= *M. canis* (MCA 01); MGY= *M. gypseum* (MGY 42); TME = *T. mentagrophytes* (TME 16*); TRU= *T. rubrum* (TRU 50). MIC^{1} corresponds to MIC without addition of commercial ergosterol; MIC^{2}, MIC^{3}, MIC^{4}, and MIC^{5}, correspond to MIC with addition of ergosterol at the concentration of 50 μg mL^{-1}, 100 μg mL^{-1}, 150 μg mL^{-1}, and 200 μg mL^{-1}, respectively. For *Candida* spp., readings 1 and 2 were performed after 2 and 5 days of incubation, respectively; for dermatophytes, readings 1 and 2 were performed after 4 and 7 days of incubation, respectively.

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