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Profile of susceptibility *in vitro* of *Trichosporon asahii* and *Trichosporon inkin* strains against cyclic imides

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In vitro *susceptibility testing of* Trichosporon asahii *(13 strains) and* T. inkin *(13 strains) against cyclic imides (succinimides, naphtalimides and maleimides) in concentrations of 200 to 6,25 µg/mL was performed according to the diffusion agar method. By the results obtained, the antifungal activity of the cyclic imides: 3,4-dichloro-*N*-phenyl-maleimide; 3,4-dichloro-*N*-phenyl-ethyl-maleimide and 3,4-dichloro-*N*-phenyl-propyl-maleimide (50 µg/ mL) over* T. asahii *and* T. inkin *was important and it may be helpful in showing perspectives in a search for new antimicotic products.*

Uniterms
- Antifungals
- *Trichosporon asahii*
- *Trichosporon inkin*
- Cyclic imides

INTRODUCTION

A taxonomic revision proposed by Guého *et al.* (1992) showed that the genus *Trichosporon* consists of six human pathogenic species: *T. asahii, T. inkin, T. mucoides, T. cutaneum, T. asteroides* and *T. ovoides*. These species are causative agents of mucous-associated, systemic mycosis, and superficial infection, including white piedra (Guého *et al.*, 1994; Therizol-Ferley *et al.,* 1994; Pontes *et al.*, 2002a, b, c; Jahagirdir *et al.*, 2002).

In therapeutics, the amphotericin B is not consistently active over the *Trichosporon* species. The fluconazole seems to be more effective and the use of a combined therapy has been recommended due to the variable activity of the amphotericin B and also due to its nefrotoxic effects (Walsh *et al.*, 1990; Anaissie *et al.,* 1992). The great concern is about the synthetic compounds, specially those analogs or those which are originated from secondary metabolites isolated from plants.

Calixto *et al.* (1984) could isolate an alkaloid of leaves from *Phyllanthus sellowianus* (Euphorbiaceae) and Tempesta *et al.* (1988) discovered the structure and named it phyllanthimide. It has been used as a model to the synthesis of analog compounds just like the cyclic imides. Some these compounds presented are biological active (Andricopulo *et al.*, 1996; Correa *et al.,* 1997; Cruz *et al.*, 1996; Aquino *et al.* 2003). For the reasons showed above, some new alternatives to diagnose and treat infections caused by the *Trichosporon* species are necessary. The objective of this study was to evaluate the profile of susceptibility *in vitro* of *T. asahii* and *T. inkin* strains against cyclic imides.

MATERIAL AND METHODS

Cyclic imides

A total of ten synthetic analogs were selected, cyclic imides, natural phyllanthimide alkaloid derivatives isolated from *P. sellowianus* (Euphorbiaceae). The cyclic imides:

two naphtalimides, three succinimides and five maleimides (Figure 1) were synthesized in the Núcleo de Investigação Químico-Farmacêutica (NIQFAR) from the Universidade do Vale do Itajaí, Santa Catarina – SC, Brazil (Cechinel Filho *et al.*, 2003).

Each cyclic imide was diluted using dimethylsulfoxide (DMSO) (0,3 mL) and sterile distilled water (q.s.q. 3 mL). The final concentration of the solutions ranged from of 200 to 6,25 µg/mL (Cleeland; Squires, 1991; Dantas *et al.*, 2000; Belém, 2002).

Microorganisms

A total of twenty six human pathogenic *Trichosporon* strains were used in the study: 13 strains of *T. asahii* and 13 of *T. inkin*. Reference isolates were *T. asahii* CBS 2479 and *T. inkin* CBS 5585 which were also quality control isolates. All other strains were obtained from clinical material (skin, nails, blood, and hair) from patients suffering from deep and superficial infections and from colonization.
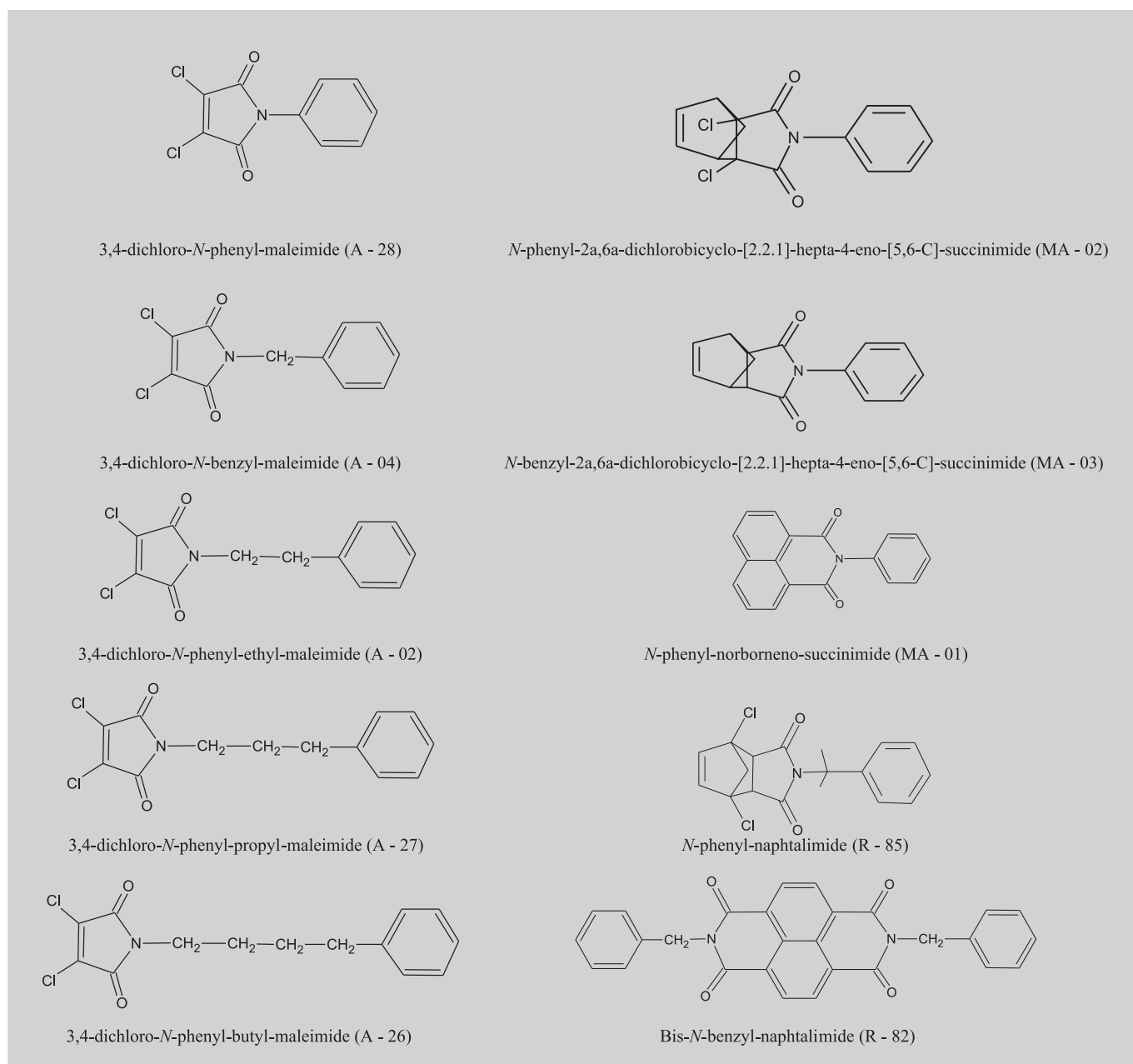

3,4-dichloro-*N*-phenyl-maleimide (A - 28)

N-phenyl-2a,6a-dichlorobicyclo-[2.2.1]-hepta-4-eno-[5,6-C]-succinimide (MA - 02)

3,4-dichloro-*N*-benzyl-maleimide (A - 04)

N-benzyl-2a,6a-dichlorobicyclo-[2.2.1]-hepta-4-eno-[5,6-C]-succinimide (MA - 03)

3,4-dichloro-*N*-phenyl-ethyl-maleimide (A - 02)

N-phenyl-norborneno-succinimide (MA - 01)

3,4-dichloro-*N*-phenyl-propyl-maleimide (A - 27)

N-phenyl-naphtalimide (R - 85)

3,4-dichloro-*N*-phenyl-butyl-maleimide (A - 26)

Bis-*N*-benzyl-naphtalimide (R - 82)

FIGURE 1 - Imides cyclic: maleimides, succinimides and naftalimides.

The present protocol was evaluated and approved with no ethical restrictions by the Ethics Committee of the Centro de Ciências da Saúde from the Universidade Federal da Paraíba (UFPB).

The mycological study was carried out at the Laboratório de Micologia of the Departamento de Ciências Farmacêuticas and the *Trichosporon* species were identified according to morphology analysis, followed by physiologic and biochemical profiles (Guého *et al.*, 1992; Guého *et al.*, 1998).

Inoculum

The inoculum was prepared by subculture each isolate in Sabouraud dextrose agar® (ASD) (DIFCO, Detroit, U.S.A) at 28-30° C for 48 hours before the experiment. The inoculum suspension was prepared by picking colonies and suspending them in saline sterile solution (0,85 %). The cell density of the suspension was adjusted to 2.5 x 10^6 UFC/mL.

Microbiological assays

In vitro susceptibility testing of *Trichosporon* species against cyclic imides was performed according to the diffusion agar method (Bauer *et al.*, 1966; Dantas *et al.*, 2000; Belém, 2002). In sterile Petri plates (90 X 15 mm), as added 20 mL of ASD previously fused and cooled in 45 to 50 °C and to each strain 1 mL of the inoculum previously prepared. After homogenization and solidification, some cavities were made by using sterile glass cannulas (6 mm of diameter). Aliquots of 50 µL of the cyclic imides solutions and of ketoconazole (200 µg/mL) control drug (Jansen Pharmaceutica, Titutsville, NJ, U.S.A) was dispensed into each cavity, followed by incubation at 28-30 °C for 48 h.

The criteria used for the definition of susceptibility to cyclic imides was the presence of an inhibition zone with a diameter bigger or equal to 10 mm around the cavity.

The strains were considered resistant when this inhibition zone was less than 10 mm of diameter.

Inoculum was removed and subcultured 28-30 °C for 48 h to check viability and purity on SDA plates. Possible interference of DMSO over the growth of the strains were evaluated adding an aliquot (50 µL) of solvent. Absence of interference was considered when there was not any inhibition zone around the cavity. Each experiment was performed in triplicate, independently. The susceptibility was determined by an arithmetic mean from the inhibition zone halos obtained in the experiments.

RESULTS AND DISCUSSION

The profile of susceptibility of the *T. asahii* and *T. inkin* strains against ten cyclic imides in concentrations of 200 to 6,25 µg/mL were diversified. All the strains were resistant to two succinimides (MA - 01 and MA - 03) and to one naphtalimide (R - 82) in all concentrations tested with an inhibition zone halo inferior to 10 mm diameter. The susceptibility of the twenty six *Trichosporon* spp. strains against seven cyclic imides (five maleimides, one succinimide and one naphtalimide) was observed in concentrations of 200 at 25 µg/mL (Tables I and II).

Cyclic imides are organic compounds formed by the group CO-N(R)-CO- being R a hydrogen atom from the group alquila or arila. Maleimides, succinimides, naphtalimides, citraconimides and derivatives, are examples of cyclic imides subclasses (Cechinel Filho *et al.*, 1995; Andricopulo *et al.*, 1998; Corrêa *et al.*, 1997). Lately, these compounds have called the attention of the scientific community mainly, due to their therapeutic potentialities as antispasmodics (Cechinel Filho *et al.*, 1995), analgesics (Andricopulo *et al.*, 1998; Corrêa *et al.*, 1997), antibacterial (Cruz *et al.*, 1996) and antifungal (Dantas *et al.*, 2000; Belém, 2002; Aquino *et al.*, 2003). The sensibility of the *T. asahii* and *T. inkin* strains against the cyclic imides groups, especially to maleimides, is an important event to notice.

Cechinel Filho *et al.* (1994) informed that in general, maleimides derivatives show an activity which is approximately 30 times higher than the succinimides against *Escherichia coli* and *Staphylococcus aureus*, suggesting that the double bond of the imido ring is a very important fact related to this activity.

Cremlyn and Nunes (1987) have also demonstrated that the sulphonyl-maleimides were much more active than the sulphonyl-succinimides against *Aspergillus niger, A. glauco, Fusarium culmorum* and *Trichoderma viride* fungi. In this study, 92 % of the *T. asahii* strains and 77 % of the *T. inkin* strains were inhibited by 3,4-dichloro-*N*-benzyl-maleimide (A - 04) in a concentration of 25 and 50 µg/mL respectively. Therefore the compounds of 3,4-dichloro-phenyl-maleimide (A – 28) and 3,4-dichloro-*N*-phenyl-ethyl-maleimide (A – 02) and 3,4-dichloro-*N*-phenyl-propyl-maleimide (A – 27) in a concentration of 50 µg/mL have inhibited the growth of 100 % of the *T. asahii* strains and 92 % of the *T. inkin* strains. *T. asahii* strains (54%) presented resistance to maleimides A - 04, A - 02, A - 28 and A-26. These results were compatible with those observed by Dantas *et al.* (2000). According to him, the maleimides which presented higher activity against *Candida albicans* strains were 3,4 dichloro-*N*-benzyl-

TABLE I – Profile of susceptibility *in vitro T. asahii* strains against cyclic imides

Cyclic imides[a]	Concentration less (µg/mL)	*T. asahii* strains												
		CBS[b] 2479	36	129	130	152	162	168	293	781	856	912	1096	5010
		Inhibition zone diameter (mm)												
A-04	50	11	10	10	10	12	11	0	10	11	12	10	11	10
A-02	50	15	14	12	13	15	13	0	12	10	15	15	10	13
A-28	50	11	10	10	10	11	11	0	10	10	10	10	10	10
A-27	50	10	10	0	10	12	12	0	12	10	10	11	11	11
MA-2	100	11	0	10	10	12	0	0	0	0	11	0	10	10
A-26	200	0	11	0	0	12	0	0	14	14	15	0	11	11
R-85	200	15	0	0	0	11	0	17	0	0	18	0	10	11
Control drug [c]	200	18	18	15	16	18	15	10	20	17	18	18	14	18

[a] A-04: 3,4-dichloro-*N*-benzyl-maleimide; A-02: 3,4-dichloro-*N*-phenyl-ethyl-maleimide; A-28: 3,4-dichloro-*N*-phenyl-maleimide; A-27: 3,4-dichloro-*N*-phenyl-propyl-maleimide; MA-2: *N*-phenyl-2a,6a,-dichlorobicyclo-[2.2.1]-hepta-4-eno-[5,6-C]-succinimide; A-26: 3,4-dichloro-*N*-phenyl-butyl-maleimide; R-85: *N*-phenyl-naphtalimide. [b] CBS: Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. [c] Control drug: Ketoconazole (200 µg/mL).

TABLE II – Profile of susceptibility *in vitro T. inkin* strains against cyclic imides

Cyclic imides [a]	Concentration less (µg/mL)	*T. inkin* strains												
		CBS[b] 5585	21	39	40	66	148	155	397	618	775	1001	1095	2726
		Inhibition zone diameter (mm)												
A-4	25	13	14	13	13	11	13	10	12	0	0	0	10	11
A-2	50	12	10	12	11	10	11	12	17	12	11	10	10	10
A-28	50	13	12	12	12	11	12	10	14	10	11	10	10	10
A-27	50	12	12	12	14	12	13	13	14	10	10	10	10	10
MA-2	100	11	12	0	10	0	10	0	12	10	0	10	0	0
A-26	100	11	11	11	11	10	11	0	11	10	0	10	11	0
R-85	200	16	13	12	0	0	11	0	20	12	0	12	14	0
Controlle drug [b]	200	21	20	21	21	18	19	20	22	18	21	16	21	18

[a] A-04: 3,4-dichloro-*N*-benzyl-maleimide; A-02: 3,4-dichloro-*N*-phenyl-ethyl-maleimide; A-28: 3,4-dichloro-*N*-phenyl-maleimide; A-27: 3,4-dichloro-*N*-phenyl-propyl-maleimide; MA-2: *N*-phenyl-2a,6a,-dichlorobicyclo-[2.2.1]-hepta-4-eno-[5,6-C]-succinimide; A-26: 3,4-dichloro-*N*-phenyl-butyl-maleimide; R-85: *N*-phenyl-naphtalimide. [b] CBS: Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. [c] Control drug: Ketoconazole (200 µg/mL).

maleimide and 3,4-dichloro-*N*-phenyl-propil-maleimide in a concentration of 100 µg/mL; with the exception of 3,4-dichloro-*N*-phenyl-ethyl-maleimide which did not inhibited the growth of new strains of *C. albicans*, two of *C. guillermondii*, one of *C. krusei*, three of *C. parapsilosis*, two of *C. stellatoidea* and two of *C. tropicalis*.

According to structure of the maleimides, the distance between the imido ring and the aromatic ring by the introduction of different substitute groups may be an important factor in relation to the activity of these compounds against the researched species. Lima *et al.* (1999) suggested that the distance between the imido ring and the aromatic ring of the *N*-phenyl-alquil-3,4-dichloro-maleimide influenced the antifungal action, that is, it was more effective especially against *Rhodotorula rubra* and *Cryptococcus neoformans* in a concentration of 50 µg/mL. They also observed that the introduction of two chlorine atoms in the imido ring did not affect the antifungal activity. Cruz *et al.* (1996) also suggested that this was an important factor in the antibacterial activity of *N*-aryl-

dichloromaleimide and *N*-aryl-naphtalimide against *Salmonella typhimurium, E. coli* and *S. aureus*.

Corrêa *et al.* (1998), searching about a possible mechanism of action of the cyclic imides, observed that they did not have any effect against *Neurospora crassa*, suggesting that they did not act by inhibiting a biosynthesis. Therefore, the cyclic imides tested by them were active against *Trichophyton rubrum, T. mentagrophytes* and *Microsporum canis*. Further studies about the mechanism of action of these cyclic imides will be very interesting to check if such compounds, for example, act at the level of the ergosterol biosynthesis.

The profile of susceptibility *in vitro* of the *Trichosporon* species studied against the succinimides and napthalimides derivatives decreased when compared to the maleimidics. The least concentrations of *N*-phenyl-2a,6a-dichlorobicyclo-[2.2.1]-hepto-4-eno-[5,6-C]-succinimide (MA -02) and *N*-phenyl-naphtalmide (R – 85) were of 100 and 200 µg/mL respectively. Belém (2002) observed that these two named compounds were also active but in lower concentrations respectively 50 and 100 µg/mL against 90% of the *M. furfur* strains. Dantas *et al.* (2000) observed that species of *Candida* were resistant to such compounds in concentrations of 6.25 and 200 µg/mL. This behavior may be possibly related to the lack of a double bond present in the imido ring, in the maleimides which produced the inhibition of growth of microorganisms (Cruz *et al.,* 1996; Lima *et al*., 1999).

It was interesting to observe that the structure of the phyllanthimide, an active naturally occurring alkaloid which follows the recommendations of the folk medicine, was used as model to the synthesis of compounds, particularly to 3,4-dichloro-*N*-phenyl-maleimide, 3,4-dichloro-*N*-phenyl-ethyl-maleimide and 3,4-dichloro-*N*-phenyl-propyl-maleimide that presented activity in a concentration of 50 µg/mL against the strains of *T. asahii* and *T. inkin*. The perspectives in the search for natural and/or synthetic products which can be used in the production of antimicotics can be broadened.

RESUMO

Perfil de sensibilidade *in vitro* de cepas de *Trichosporon asahii* e *Trichosporon inkin* frente a imidas cíclicas

O perfil de sensibilidade in vitro *de* Trichosporon asahii *(13 cepas) e* T. inkin *(13 cepas) frente a imidas cíclicas (naftalimidas, succinimidas e maleimidas) nas concentra-ções de 6,25 a 200 µg/mL foi realizado pelo método de difusão em agar. Pelos resultados obtidos, a atividade antifúngica das imidas cíclicas: 3,4-dicloro-*N*-fenil-maleimida; 3,4-dicloro-*N*-fenil-etil-maleimida e 3,4-dicloro-*N*-fenil-propil-maleimida (50 µg/mL) sobre* T. asahii *e* T. inkin *foi importante e pode abrir perspectivas na busca de novos produtos antimicóticos.*

UNITERMOS: Antifúngicos. Trichosporon asahii. Trichosporon inkin. *Ímidas cíclicas*.

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