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Antifungal Activity of Hydrochloride Salts of Tylophorinidine and Tylophorinine

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The antimicrobial efficacy of two phenanthroindolizidine alkaloids, tylophorinidine hydrochloride (TdnH) and tylophorinine hydrochloride (TnnH), isolated from the plant *Tylophora indica* (local name, Antamul) was evaluated. These were screened for *in vitro* antifungal and antibacterial activities. Both compounds exhibited potent antifungal activity displaying minimum inhibitory concentrations (MIC) in the range of 2-4 µg/mL for TdnH and 0.6-2.5 µg/mL for TnnH against *Candida* species.

Keywords: *Tylophora indica*, Antimicrobial, Phenanthroindolizidine, Antifungal, Tylophorinidine, Tylophorinine.

Phenanthroindolizidine alkaloids are the distinctive constituents of *Tylophora* species from the family Asclepiadaceae. Traditionally, *T. indica* has been used for the treatment of asthma, jaundice, inflammation and allergy [1,2]. The plant has been reported to contain alkaloids, sterols, flavanoids, wax, resins, and tannins [3]. The other reported activities include cytotoxic [4], immuno-modulatory [5a], anticancer [5b] and antiamoebic [5c]. *T. indica* is also known to have antibacterial and antifungal properties and the alkaloid isolated from *Tylophora* displayed strong antibacterial activity [3b,5d,6]. In the present work, two alkaloids, tylophorinidine and tylophorinine, were isolated as hydrochloride salts (Figure 1) from aerial parts of *T. indica* [7] and evaluated for their *in-vitro* antifungal and antibacterial potency. Both were previously reported not only from *T. indica*, but also from other species of *Tylophora* [7,8a-c]. The crude extract (CE) along with the isolated pure compounds showed considerable antimicrobial activities against several fungal, Gram-positive and -negative bacterial species. At 1mg/mL concentration the C showed inhibitory activity against *Candida* and *Aspergillus* spp. (Table 1). It also inhibited fluconazole resistant *Candida* spp., *C. glabrata* HO5; $Fluc^R$. The crude extract thus signaled the broad spectrum antifungal trait of the extract. At 1mg/mL, the crude extract could inhibit only vancomycin resistant enterococci, VRE; *Enterococcus faecium*. In these preliminary studies, both TdnH and TnnH showed

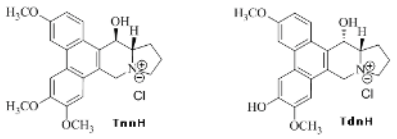

Figure 1: Hydrochloride salts of tylophorinine (TnnH) and tylophorinidine (TdnH).

broad spectrum antifungal activity against all genera of fungi under study encompassing both fluconazole resistant strains (*Candida krusei* GO3 and *C. glabrata* HO5). Like the crude extract, the compounds also displayed only weak anti-VRE activity against the bacterial test strains. However, TdnH did show some weak activity at 1 mg/mL, even against *S. aureus*.

Based on the results of the activity profile and doses in preliminary studies, MIC values were evaluated for TdnH and TnnH, along with comparative clinical antifungal agents. Both compounds showed a wide spectrum and potent inhibition of unicellular, filamentous and resistant fungal strains. The MIC of TdnH against *Candida* spp. was found to be in the range of 2-8 µg/mL and for *A. fumigatus* 8.0 µg/mL. The MIC values for TdnH were much lower than those of Fluconazole, but higher than those of Amphotericin B (Table 2). The MIC of TnnH against *Candida* spp. was in the range of 0.625-2.5 µg/mL and for *A. fumigatus* 5.0 µg/mL. TnnH was

Table 1: Antimicrobial activity of TnnH, TdnH and the crude extract of *Tylophora indica* against different test strains of microorganism by agar well diffusion assay.

Sample ID	Conc (mg/mL)	Fungal test organisms							Bacterial test organisms					
		C. albicans (I.V.)	*C. albicans* ATCC 14503	*C. krusei* GO3, $Fluc^R$	*C. krusei* GO6, $Fluc^S$	*C. glabrata* HO4, $Fluc^S$	*C. glabrata* HO5, $Fluc^R$	*A. fumigatus* (HMR)	*A. fumigatus* ATCC 16424	*Cryptococcus neoformans*	*S. aureus* 209P	*E. faecium* VRE 323	*E.coli* ATCC 35218	*E.coli* ATCC 25922
TdnH	0.1	20	14	14	17	11	15	13h	10h	9slh	-	-	-	-
	1	24	21	21	24	20	21	17	16	17	-	9vh	-	-
TnnH	0.1	12	15	sl	12	13	9h	sl	9	8	-	-	-	-
	1	19	22	18	16	18	17	16	14	14	11h	12h	-	-
Crude extract	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-
	1	12	-	-	10	-	10	9	9	-	-	10	-	-
AmB	0.02	20	19	15	14	20	20	16	20	22	NT	NT	NT	NT
Van	0.02	NT	NT	NT	NT	NT	NT	NT	NT	NT	14	12	NT	NT
Gen	0.05	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	15	14

'$Fluc^R$' Fluconazole resistant; '$Fluc^S$' Fluconazole sensitive; '-' no zone of inhibition; 'NA' Not applicable; 'h' hazy zone of inhibition; 'sl' slight zone of inhibition; 'NT' Not tested; 'AmB' Amphotericin B; 'Van' Vancomycin; 'Gen' Gentamicin.

Table 2: Comparative antifungal activity of the TnnH and TdnH

Test organisms	MIC (µg/mL) of compounds			
	TnnH	TdnH	AmB	Fluconazole
C. albicans (I.V.)	0.625	2	0.313	>256
C. albicans ATCC 14503	0.625	2	0.313	>256
C. krusei GO3, $Fluc^R$	0.625	4	0.313	64
C. krusei GO6, $Fluc^S$	0.625	2	0.313	2
C. glabrata HO4, $Fluc^S$	2.5	8	0.313	8
C. glabrata HO5, $Fluc^R$	1.25	4	0.313	>256
A. fumigatus (HMR)	5	8	0.625	>256
A. fumigatus ATCC 16424	NT	8	NT	>256

For each test organism n=2, NT: Not tested, AmB: Amphotericin B

found to have a broad spectrum of activity and to be a potent antifungal compound. Its activity was far superior to that of Fluconazole in the *in-vitro* studies. Its antifungal activity was comparable with that of Amphotericin B, especially against *C. albicans* and *C. krusei* strains. This is the first report of the broad-spectrum, potent antifungal activity of TdnH and TnnH. As the chemical class of these compounds is different from those of existing antifungal compounds used in clinics or which are in the pipeline, we envisage that these two could be potential scaffolds for further antifungal lead development.

Experimental

Test species: The different bacterial and fungal strains were procured from the American Type culture collection (ATCC), Manassas, USA, and from the strains collection of Hoechst Research Center, Mulund, Mumbai-India. The bacterial test strains included *Staphylococcus aureus* 209P, *Enterococcus faecium* R-2; VRE, *Escherichia coli* ATCC 35218 and *E. coli* ATCC 25922, and the fungal test strains included *Candida albicans* (I.V.), *C. albicans* ATCC 14503, *C. krusei* GO3; $Fluc^R$, *C. krusei* GO6; $Fluc^S$, *C. glabrata* HO4; $Fluc^S$, *C. glabrata* HO5; $Fluc^R$, *Cryptococcus neoformans*, *Aspergillus fumigatus* (HMR) and *A. fumigatus* ATCC 16424.

Antimicrobial activity

Whole cell agar well diffusion assay: Antimicrobial activity was determined by the whole cell agar well diffusion assay [8d]. The bacterial test strains were grown on Tryptone Soya agar and the fungal test strains on Sabouraud agar plates. Wells were punched into the agar medium and different concentrations of the compounds were loaded into the wells. Amphotericin B (20 µg/mL) and Vancomycin (20 µg/mL) were the standards used against fungal and bacterial test strains, respectively. The plates were incubated at 37°C for 24 h. The results were recorded as the diameter of the zone of inhibition around each well, expressed in mm.

Minimum inhibitory concentration estimation: MIC values of TdnH, TnnH, Amphotericin B and Fluconazole were determined by the NCCLS (CLSI) Macrobroth dilution method for yeasts (M27-A2) and filamentous fungi M38-A [8e,f]. The microbes were grown on Sabouraud agar slants at 37°C for 48 h and the inoculum was prepared in sterile saline. Optical density of the suspension was adjusted to 1.0 at 600 nm so as to obtain 10^8 colony forming units (cfu)/mL for *Candida* species. The final working inoculum was 0.5 X 10^4 cfu/mL. For *Aspergillus* species, the inoculum was prepared in sterile saline containing 0.01% Tween 80 for uniform distribution of the spores. The percent transmittance was adjusted between 80-82% at 530 nm so as to produce 10^6 spores/mL. The final working inoculum was 0.5 X 10^4 spores/mL. Based on the results of the agar well diffusion assays, TdnH was suitably diluted in the concentration range of 0.5 – 256 µg/mL, and TnnH in the range of 0.08 – 40 µg/mL. The MIC assay tubes were incubated at 37°C for 48 h. Appropriate controls (positive, negative, solvent, and medium) were used in the assay, and the tubes were observed for growth of test culture in terms of turbidity visible to the naked eyes after 48 h of incubation. The MIC values of the compounds were recorded.

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