Viriditoxin, an antibacterial substance produced by mangrove endophytic fungus *Paecilomyces variotii*

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In the course of the search for biologically active compounds, the purpose of this study was to isolate, structure characterization and antibacterial activity of a substance produced by mangrove endophytic fungus *Paecilomyces variotii* FEL 32. Viriditoxin was isolate by column chromatography and identified in according to mass spectrum and nuclear magnetic resonance data. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of viriditoxin were evaluated on pathogenic bacteria of clinical relevance at 32-0.06 μg/ml concentration range. Our results pointed that viriditoxin presented MIC values between 0.5 and 2 μg/ml, and MBC values between 1 and 8 2 μg/ml. The clinical isolate *Staphylococcus aureus* UFPEDA 614 was the most sensible presenting a MIC of 0.5 μg/ml followed for *Enterococcus* sp. UFPEDA 620 presenting a MIC of 2 μg/ml and MBC of 8 μg/ml. For the clinical isolates of *S. aureus* negative coagulase UFPEDA 628, 629 and 630, the values of MIC and MBC were 1, 2 and 2 μg/ml, respectively.

Keywords viriditoxin; *Paecilomyces variotii*; endophytic fungi; mangrove; naphthopyranones

1. Introduction

Endophytes are organisms which spend all or part of their lifecycle inter and/or intracellularly colonizing organs and healthy tissues of plants, typically without causing apparent disease symptoms [1]. They are recognized to producing a great variability of metabolites which help the host plant to survive to the environmental conditions and infections caused for pathogens [2]. The endophytic fungi are excellent synthesizers of secondary metabolites with wide biological activity and play fundamental role in the sustainability of the ecosystems and biodiversity [3, 4]. As consequence, a lot of attention has been given to the potential of exploitation of these fungi for the production of novel antibiotics [5].

The number of secondary metabolites produced by endophytic fungi is larger than that of any other endophytic microorganism class and many endophytic fungi have been reported to produce antimicrobial substances, such as *Colletotrichum* sp., *Xylaria* sp., *Pestalotiopsis* sp., *Paecilomyces* sp., *Phomopsis* sp. and *Phoma* sp. [6-8]. The endophytic fungi also were found in estuarine environment but these studies are essentially about the ecologic relationship between fungi and host plant, few papers investigate the biotechnological and biological potential of these microorganisms [9-14].

Mangroves are intertidal forested wetlands confined to tropical and subtropical regions. These ecosystems are a dynamic transition zone between terrestrial and marine habitats. Mangrove forests are biodiversity hotspots for marine fungi and constitute the second largest ecological group of these microorganisms [15]. Mangrove endophytic fungi have been proved to be a well established source for structurally diverse and biologically active secondary metabolites [16].

Microbial resistance has increased in both Gram-positive and Gram-negative bacteria as well as some yeasts and presents a serious threat to the antimicrobial treatment of infectious diseases. Bacterial species such as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterobacter* sp. cause the majority of hospital infections and become resistant to the effects of antibacterial drugs [17].

Multidrug resistant *Staphylococcus* cause significant morbidity and mortality and are leading of nosocomial infections. Meanwhile, methicillin-resistant *Staphylococcus aureus* (MRSA) also spreads in the community, where virulent strains infect children and young adults who have no predisposing risk factor [18].

In the battle against multidrug resistance, it's necessary new alternatives to search broad-spectrum antibiotics. Based in this fact, the objective of our work was isolate, structural characterization and antibacterial activity of a substance produced by mangrove endophytic fungus *Paecilomyces variotii* against pathogenic bacteria of clinical relevance.

2. Material and Methods

2.1. Endophytic fungus

The endophytic fungus *Paecilomyces variotii* FEL 32 was isolated from the healthy leaves of *Laguncularia racemosa* (L.) Gaertn. (White mangrove, Combretaceae) collected from estuary of the Paripe River, Ilha de Itamaracá, Pernambuco, Brazil. The fungal identification was performed by observation of the macro and microscopy characteristics as specific literature [19]. The fungal strain was maintained on potato dextrose agar (PDA) slants at ± 4°C until further use.

2.2. Culture conditions and fermentation

Firstly, *P. variotii* FEL 32 were grown in PDA culture medium at ± 30°C for 3 days. Subsequently, a pre-inoculum was prepared from 2 ml of conidial suspension ($5x10^4$ spores/ml) and inoculated into 125 ml Erlenmeyer flask containing 20 ml of Sabouraud dextrose broth (SDB) and incubated at ± 28°C for 48 hours under agitation (200 rpm). For the solid-state fermentation, the pre-inoculum was transferred into 500 ml Erlenmeyer flask containing 100 g of Japanese rice (Momiji™) as solid substrate during 18 days at temperature of ± 28°C under static conditions.

2.3. Obtainment of the crude extract

At the end of fermentation, the secondary metabolites produced by *P. variotii* FEL 32 were extracted with 250 ml of $CHCL_3$/MeOH (1:1 v/v) solvent system by sonication during 40 minutes. The solvent was separated of the solid substrate by vacuum filtration and concentrated in rotary evaporator at temperature of ± 45°C. The concentrated extract was stored in a vacuum desiccator until constant weight. Further, the crude extract was previously treated with 100 ml of n-hexane in order to remove apolar fraction and solubilized in 500 ml of EtOAc, obtaining the organic phase. The monitoring of the antimicrobial activity of the organic phase was performed the disk diffusion test toward Gram-positive bacterium *Bacillus subtilis*.

2.4. Pre-purification of the crude extract

The organic phase (4 g) was responsible by antimicrobial activity and submitted to column chromatography on silica gel 60 using $CHCl_3$:MeOH (10:0→10:1 v/v) gradient, yielded 9 fractions. Biochromatography using *B. subtilis* reveals that only the fraction number 5 (1.4 g) showed positive antimicrobial activity, being resubmitted to column chromatography on silica gel 60 using $CHCl_3$:MeOH (10:0→10:3 v/v) gradient, yielding 9 fractions, where the fractions of number 3, 4, 5 and 6 showed antimicrobial activity. These fractions were combinated on a unique volume and subjected to column chromatography using $CHCl_3$:Acetone:MeOH (10:1:0→10:0:3 v/v) gradient, yielding 30 fractions. All the 30 fractions were chromatographed on silica gel plates (Si250F, 20 x 20 cm) with fluorescent indicator (Mallinckrodt Baker Inc., Phillipsburg, Nova Jersey, USA) using CHCl3:MeOH (10:3 v/v), grouped according to chromatographic profile and tested toward *B. subtilis*.

2.5. Isolation of viriditoxin

The grouped fractions were concentrated and separated in two categories: soluble and insoluble in Et_2O. Viriditoxin was isolate from fraction soluble in Et_2O and your structure elucidation was performed by mass spectroscopy (MS) on a AccuTOF JMS-T100 equipped with direct analysis in real time (DART) ion source (JEOL) and nuclear magnetic resonance (NMR) using a Brunker AV-400 instrument at 400 MHz and 100 MHz for the ^1H and ^{13}C spectra, respectively. The NMR and MS spectra were measured at Faculty of Pharmaceutical Sciences, Hoshi University, Tokyo, Japan.

2.6. Test microorganisms and antibacterial assay

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of viriditoxin were determinate in according to M7-A6 protocol recommended by CLSI towards the following pathogenic bacteria of clinical relevance: *Micrococcus* sp. UFPEDA 610, *Micrococcus* sp. UFPEDA 611, *Staphylococcus aureus* UFPEDA 614, *S. aureus* UFPEDA 618, *S. aureus* UFPEDA 619, *Enterococcus* sp. UFPEDA 620, *S. aureus* negative coagulase UFPEDA 628, *S. aureus* negative coagulase UFPEDA 629 and *S. aureus* negative coagulase UFPEDA 630. Informations about the source of each clinical isolate were expressed on the table 1. All bacteria were provided by Microorganisms Culture Collection, Department of Antibiotics, Federal University of Pernambuco, Brazil. Viriditoxin was tested at 32-0.06 μg/ml concentration range and the MIC was defined as the lowest concentration of the substance that inhibited the visible growth of a test microorganism.

Table 1 Clinical isolates used for antibacterial assay of viriditoxin.

Microorganism	**Source**
Micrococcus sp. UFPEDA 610	Uroculture
Micrococcus sp. UFPEDA 611	Uroculture
Staphylococcus aureus UFPEDA 614	Hemoculture
Staphylococcus aureus UFPEDA 618	Hemoculture
Staphylococcus aureus UFPEDA 619	Hemoculture
Enterococcus sp. UFPEDA 620	Ulcer
Staphylococcus sp. negative coagulase UFPEDA 628	Hemoculture
Staphylococcus sp. negative coagulase UFPEDA 629	Uroculture
Staphylococcus sp. negative coagulase UFPEDA 630	Hemoculture

UFPEDA: Microorganism Culture Collection, UFPE, Pernambuco, Brazil.

3. Results and Discussion

The *Paecilomyces* genus includes many species able to produce secondary metabolites belongs to different chemical groups with wide biological activity such as leucinostatins A, D, H and K [20-22], paeciloquinones [23], ergosterol [24], brefeldin A [25], paecilosetin [26], paecilaminol [27], paecilodepsipeptide A [28], paecilin A and B [29].

The major substance resultant of the solid-state fermentation on Japanese rice by endophyte *P. variotii* FEL 32 and responsible by the antibacterial activity was obtained from the fraction soluble in ether as a yellow powder, yielding 4 mg and identified as viriditoxin. Structural characterization by ^{1}H and ^{13}C-NMR data shows that this substance is a dimer (6,6' binaphtho-α-pyranone) of molecular formula $C_{34}H_{30}O_{14}$ and molecular weight 662. Moreover, mass spectrum of viriditoxin (fig. 1) was similar to the observed in another study confirming your identification [30]. Viriditoxin was firstly isolated and elucidated from mycelium of *Aspergillus viridi-nutans* NRRL 4365 [31] and posterior studies from *A. viridi-nutans* NRRL 576 and *A. viridi-nutans* NRRL 4078 [32].

Monomeric and dimeric forms of the naphthopyranones usually are presented as a yellow pigments resultant of the secondary metabolism of some species from *Aspergillus*, *Fusarium*, *Paecilomyces* and eventually from plants [33-35]. *P. variotii* is recognized to produce naphthopyranones, specially viriditoxin and derivatives, however, our study is the first report of the production of viriditoxin by endophytic *P. variotii* from estuarine environmental.

The literature reveals that some naphthopyranones have anticancer, antibacterial, antifungal and anti-insecticidal properties [36-38]. Fungal strain *Aspergillus niger* IBF-E003 from *Cynodon dactylon* leaves produces 04 naphthopyranones with antimicrobial activity against *B. subtilis*, *Pseudomonas fluorescence*, *Trichophyton rubrum* and *Candida albicans* [39].

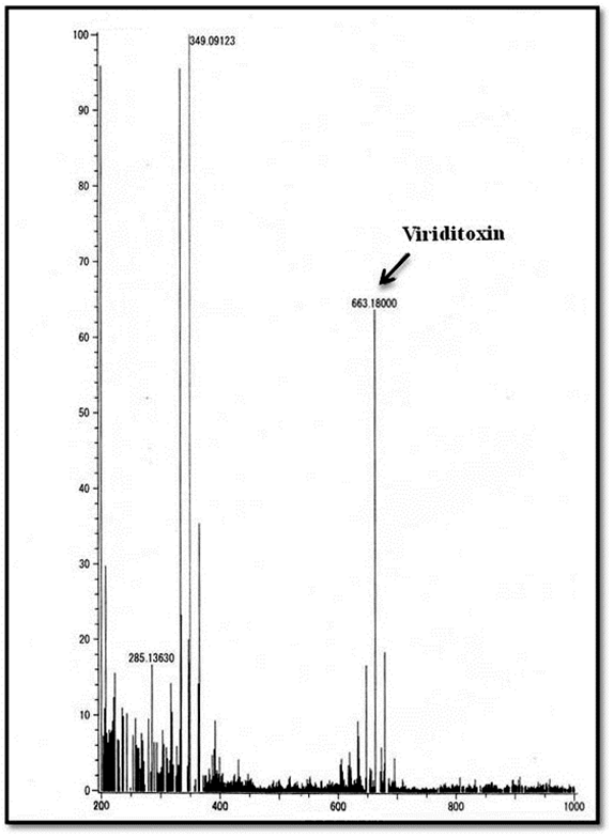

Fig. 1 Mass spectrum of viriditoxin.

In our work, viriditoxin was tested against pathogenic bacteria and the results of the MIC and MBC values are showed on the table 2. All clinical isolates were inhibited for viriditoxin, with MIC of 0.5-2 µg/ml and MBC of 2-8 µg/ml. The clinical isolate *S. aureus* UFPEDA 614 was the most sensible with MIC of 0.5 µg/ml, following by *Micrococcus* sp. UFPEDA 610 and 611 were inhibited with MIC of 2 and 1 µg/ml, respectively. *Enterococcus* sp. UFPEDA 620 was inhibited with MIC of 2 µg/ml, but the MBC was 8 µg/ml. For the clinical isolates from *S. aureus* negative coagulase UFPEDA 628, 629 and 630 the MIC were 1, 2 and 2 µg/ml and same results for MBC values, respectively. The results here described confirm the antibacterial activity of viriditoxin mainly for Gram-positive bacteria. The MIC and MBC values obtained were lowest than other study with viriditoxin isolated from *Aspergillus* sp. MF6890 [40].

Table 2 MIC and MBC values of viriditoxin against pathogenic bacteria.

Test microorganism	MIC (µg/mL)	MBC (µg/mL)
Micrococcus sp. UFPEDA 610	2	2
Micrococcus sp. UFPEDA 611	1	2
Staphylococcus aureus UFPEDA 614	0.5	1
Staphylococcus aureus UFPEDA 618	2	2
Staphylococcus aureus UFPEDA 619	1	2
Enterococcus sp. UFPEDA 620	2	8
Staphylococcus sp. negative coagulase UFPEDA 628	1	1
Staphylococcus sp. negative coagulase UFPEDA 629	2	2
Staphylococcus sp. negative coagulase UFPEDA 630	2	2

MIC: Minimal inhibitory concentrarion; MBC: Minimal bactericidal concentration.

The action mechanism of viriditoxin is well established on literature and occurs by inhibition of the polymerization of FtsZ protein, a GTPase homologue to β-tubulin and essential for bacterial celular division [41,42]. FtsZ is the most

abundant of all proteins during cell division with nearly 10.000 - 20.000 copy/bacterial cell. The first step of the cell duplication process is FtsZ polymerization, however viriditoxin blocks off this step bringing to cell death. The spectrum of viriditoxin is amazing, including strains of *S. aureus* methicilin resistant, *Enterococcus* sp. vancomicine resistant. The isolation of a antibacterial substance with target specific, due to the high degree of conservation of the FtsZ protein, becomes a excellent opportunity to the development of the novel pharmacs.

4. Conclusion

The real significance of endophytic fungi within mangrove plants remains unknown, but the results here presented confirm the importance of the search for marine microorganisms as alternative for obtainment of new antimicrobial compounds.

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