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Active pharmaceutical ingredient (api) from an estuarine fungus, *Microdochium nivale* (Fr.)

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Abstract

Various marine habitats sustain variety of bio-sources of ecological and biotech potentials. Pharmaceutical potential compound Cyclosporine A was reported from marine fungus *Microdochium nivale* associated with *Porteresia coarctata,* a marine salt marsh grass from mangrove environment distributed along the Central West Coast (CWC) of India. This study involves association of *M. nivale* with *P. coarctata* plant, fermentation conditions, purification of Cyclosporine A, chemical characterization etc. Its antifungal inhibition and MIC (Minimum inhibitory concentration) against *Aspergillus* strains (*A. niger*, *A. japonicus, A. fresenii*), yeasts and dermatophytes (*Candida* sp., *Cryptococcus neoformans, Trichophyton mentagrophytes*, *T. tonsurans*, *T. violaceum*, *Microsporium gypsum* and *Fusarium* sp.) were evaluated. However, the MIC against *A. japonicus, C. neoformans, Candida* sp. and *T. tonsurans* were confirmed to be as low as 12.5-25 mg disc^{-1}. The antifungal properties of Cyclosporine A against *Aspergillus* species, yeast and dermatophytes revealed that Cyclosporine A would be a potential compound for life threatening diseases caused by above fungi in both human and animals. Furthermore, we have reported herewith another source of Cyclosporin A derived from filamentous fungus, *M. nivale*. occurring in marine environment.

Key words

Microdochium nivale, Cyclosporine A, Antifungal, Estuarine environment

Introduction

The cyclosporine are a group of over 25 closely related cyclic undecapeptides produced as secondary metabolites by certain species of filamentous fungi. Cyclosporine A is a drug with broad spectrum pharmaceutical properties such as antifungal, antiparasitic, anti-inflammatory, Keratoconctivitis sicca (KCS) and immunosuppressive activity (Stiller and Opelez, 1991; Rahacek and De-Xiu, 1991; Laibowitz *et al.*, 1993). Cyclosporine A is a potent immunosuppressant that has significantly contributed to the progress in organ transplantation such as kidney, heart, liver, lungs and other organs in the human body. Cyclosporine activity as an immunosuppressant which acted mainly on T-lymphocytes, showed anti-inflammatory effects and was practically devoid of toxicity (Borel *et al.*, 1976, 1977). It is unique among the presently available immunosuppressive drugs in that it reversibly inhibits only some classes of lymphocytes and does not affect haemopoietic tissues (bone marrow). Cyclosporine A was introduced into clinical use in the late 1970s to reduce graft rejection after organ transplantation, a property based upon interference of the agent with lymphokine biosyntheis (Randake *et al.*, 1990; Schreiber and Crabtree, 1992). It has also been proven effective in a variety of autoimmune and related disorder.

A number of fungi from the marine environments have been reported to be important sources of bioactive compounds (Biabani and Laatsch,1998; Faulkner 2002; Bugni and Ireland, 2004; Paul *et al*., 2006). *Fusarium* genus distributed widely in soil and in an association with plants and most of the species are harmless saprobes rich in soil microbial community. These forms are known to be of a great industrial and pharmaceutical applications being rich in peptides, proteins and allergens (Singh *et al*., 2003; Varma *et al*., 2003; Khan *et al*., 2007). *Fusarium fujikuroi* is used commercially for the large production of gibberellin which has applications in agriculture and horticulture and plant growth regulator (Rademacher, 1997). The plant growth regulator jasmonates and related compounds were reported from culture filtrate of *F. oxysporum* (Miersch *et al*., 1999). *Fusarium* mycotoxins constitute a very heterogeneous group of compounds like trichothecenes (cyclic sesquiterpenoids), zearalenone, fumanisins and moniliformin (Krska *et al*., 2007). We report herein, association of *Microdochium nivale* (*Fusarium nivale*) with *P. coarctata* plant, in fermentation conditions, and its isolation, purification, bioactive potential and chemical characterization of Cyclosporine A.

Materials and Methods

Isolation of *Aspergillus* species and dermatophytes: Three strains of *Aspergillus*, were isolated from wheat bread. They were grown in mycological agar and sub-cultured for purification, following standard procedure (Chen *et al*., 1996). The taxonomic identification of fungal species were carried out by using relevant keys (Ainsworth *et al*., 1973; Domsch *et al*., 1980). Acetone extracts of pure cultures of these species were tested individually for their antifungal properties.

Commonly occurring pathogenic fungal strains of yeast (*Candida* sp. *Cryptococcus neoformans*) and filamentous fungi (*Trichophyton mentagrophytes*, *T. tonsurans*, *T. violaceum, Microsporium gypsum* and *Fusarium* sp.) were obtained from Goa Medical College, Bambolim. A colony of *Candida* species and *C. neoformans* were transferred on mycological agar (HiMedia Laboratories Ltd.) and maintained in the refrigerator at 3-5°C, for further studies. Similarly, other filamentous strains were maintained on Czapek Malt agar (HiMedia Laboratories Ltd.) slants in the laboratory at 3-5°C, for further studies.

Isolation of *Microdochium nivale:* Leaves of *P. coarctata* were collected from Mandovi estuary, Goa, India. These leaves were washed immediately after collection, with ambient seawater to remove adhering debris, and transported to the laboratory in sterile polythene bag, and were quickly transferred to the moist chamber (28±1°C). The humidity in the moisture chamber was maintained at constant by adding ~ 5ml of sterile seawater every alternate day. The white colour colony appeared on the leaves after ~15-29 days of incubation. This fungal colony was transferred onto the culture media nutrient agar (Hi-media India Ltd.) in sterile plates and further purified by repeated sub-culturing using standard procedure (Collin and Tayler, 1967).

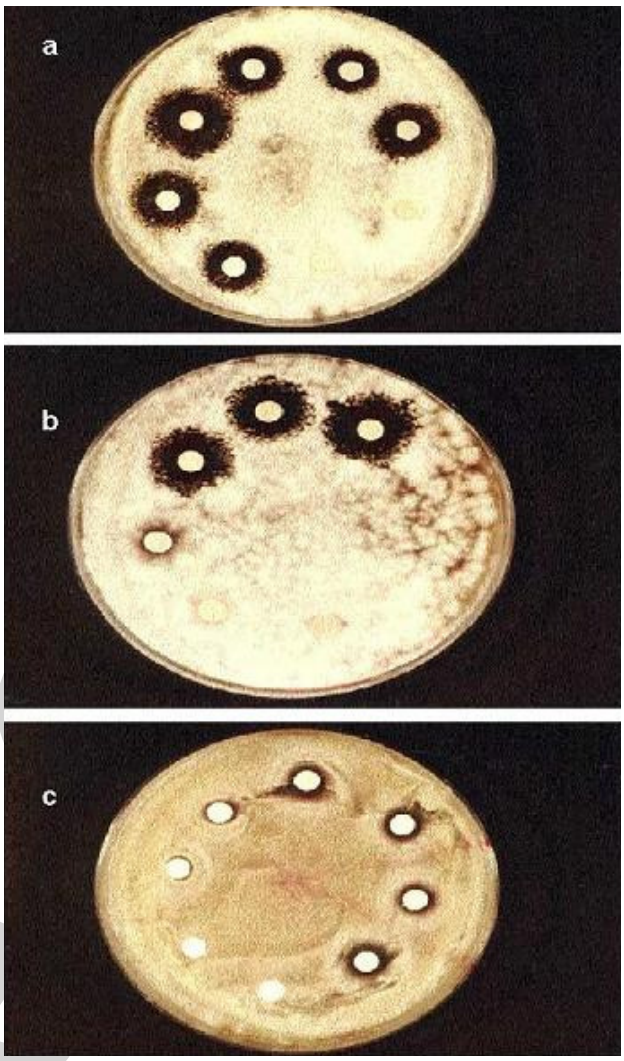

Fig. 1: MIC of Cyclosporine A. against (a) *Trichophyton tonsurans* (b) *T. violaceum* (c) *Fusarium* sp.

Microdochium nivale was grown in the nutrient broth (Hi-media Laboratories Pvt. Ltd.) with 50% seawater. The mass culture was obtained in Erlenmeyer flasks (2000 ml capacity) containing 500 ml of each production medium. The second stage inoculum (10% v/v) were inoculated and incubated for 8 days under static condition by growing it at 28 ± 1°C in the laboratory. The mycelia biomass was removed at its optimum growth and freeze-dried in a lyophilizer (Heidolph, Germany).

Antifungal activity: Antifungal activity studies were carried out using the disc diffusion method in agar plate petri dishes. Whatman (GF/F) filter paper discs of 6 mm diameter were prepared and sterilized in autoclave for ~15 min under 15 lbs pressure. The stock solution for the test was prepared by dissolving 0.25 gm of each extract in 1 ml of solvent depending upon the solubility of extracts either in methanol, acetone or dimethyl sulfoxide (DMSO). Final concentration of 250 mg $disc^{-1}$ of each stock solution was obtained by spreading 10 ml of stock

solution on the paper disc. The discs were placed in mycological agar (pH - 7.3), and plates were seeded with different fungal strains. The cultures were incubated for 24-148 hrs at room temperature, to obtain maximum growth in the culture media. Standard discs of antifungal agent Amphotericin - B (100 units $disc^{-1}$) and Nystatin (100 units $disc^{-1}$) were used to check the sensitivity. Control tests with the solvents loaded on to discs were performed in triplicate, for every bioassay of a set, in triplicate of each sample extract, and the results were expressed as average of an inhibition zone in mm.

Minimum inhibitory concentration (MIC): Inoculums were adjusted to 50 ml of dermatophytes such as *Cryptococcus neoformans*, *Candida* sp., *Microsporium gypsum*, *Trichophyton tonsurans*, *T. violaceum*, *T. mentagrophytes*, *T. rubrum*, *Fusarium* sp., and food spoilage fungi *Aspergillus niger*, *A. japonicus*, *A. fresenii,* was spread on the appropriate plates. Excess moisture from plates was allowed to absorb for ~10 min before applying discs containing different concentrations of standards and compound A extracted from *M. nivale*. For conducting bioassay studies for the MIC, the stock solutions of compound A and reference compounds Amphotericin B (Potency = ~750 $\mu g\ mg^{-1}$ and Nystatin (potency = 4800 USP unit mg^{-1}), were prepared in different concentrations by dissolving them in dimethylsulphoxide (DMSO). The concentrations were prepared by dissolving 1, 0.5 and 0.25 mg in 200 ml of (DMSO) and 0.625 and 0.312 mg in 1 ml of (DMSO). From these stock solutions, 10 ml were spread on the GF/C Whatman paper discs so as to obtain the final concentrations of 50, 25, 12.5, 6.25 and 3.12 $\mu g\ disc^{-1}$. Discs were dried at a reduced pressure in the dessicator, at 28-29°C and applied to inoculated plates. Test plates were incubated for 42-120 hrs at room temperature (28 ± 1°C) to obtain maximum growth, and the MIC of compound A and antifungal agents resulting in a clear zone of growth inhibition was determined. It was confirmed by experiments that DMSO alone had no detectable inhibitory effect on any of the test organisms.

Purification of Cyclosporin A: The entire lyophilized mass of *M. nivale* was extracted using 90% aqueous acetone at ambient temperature of 28 ± 1°C. This acetone extract was dried by condensing under reduced pressure using the Laborota 4000 (Heidolph, Germany). The crude acetone extract of *M. nivale* was partitioned successively with petroleum ether and ethyl acetate. The residue was taken as aqueous fractions by using the separating funnel. All the three fractions were screened for antifungal activity against *Aspergillus* strains causing food spoilage and dermatophytes

Table - 1: Antifungal activity of different fractions at the concentration 250 $\mu g\ disc^{-1}$

Name of fungi	PEF	EAF	AF	Nystatin	Amph.
Aspergillus fresenii	-	++	++	++	+++
Aspergillus japonicus	-	+++	++	++	+++
Aspergillus niger	-	++	+	++	+++
Cryptococcus neoformans	-	++	++	++++	+++
Candida sp.	-	++	++	+++	+++
Microsporum gypsum	-	++	++	+	++++
Trichophyton mentagrophytes	-	+++	++	++	+++
T. rubrum-	+++	+	++	+++	
T. violaceum	-	+++	++	++	+++
T. tonsurans	-	+++	++	++	++++
Fusarium sp.	-	+++	+	++	+++

PEF = Petroleum ether fraction, EAF = Ethyl acetate fraction, AF = Acetone fraction
- = No inhibition, + = 7-8 mm inhibition, ++ = 8-9 mm inhibition, +++ = 8-11 mm inhibition, ++++ = 11-13 mm inhibition

Table - 2: Minimum inhibitory concentration (MIC) of Cyclosporin A purified from *Microdochium nivale*

	Disc diffusion method		
Test organisms	**Cyclosporin A. (µg $disc^{-1}$)**	**Amphotericin B (µg $disc^{-1}$)**	**Nystatin (µg $disc^{-1}$)**
Aspergillus fresenii	25 - 50	6.25 - 12.5	-
A. japonicus	12.5 - 25	6.25 - 12.5	-
A. niger	25 - 50	6.25 - 12.5	-
Cryptococcus neoformans	12.5 - 25	3.12 - 6.25	3.12 - 6.25
Candida sp.	12.5 - 25	3.12 - 6.25	3.12 - 6.25
Trichophyton mentagrophytes	25 - 50	3.12 - 6.25	-
T. tonsurans	12.5 - 25	6.25 - 12.5	-
T. violaceum	25 - 50	6.25 - 12.5	-
Fusarium sp.	25 - 50	12.5 - 25	-

- = No inhibition

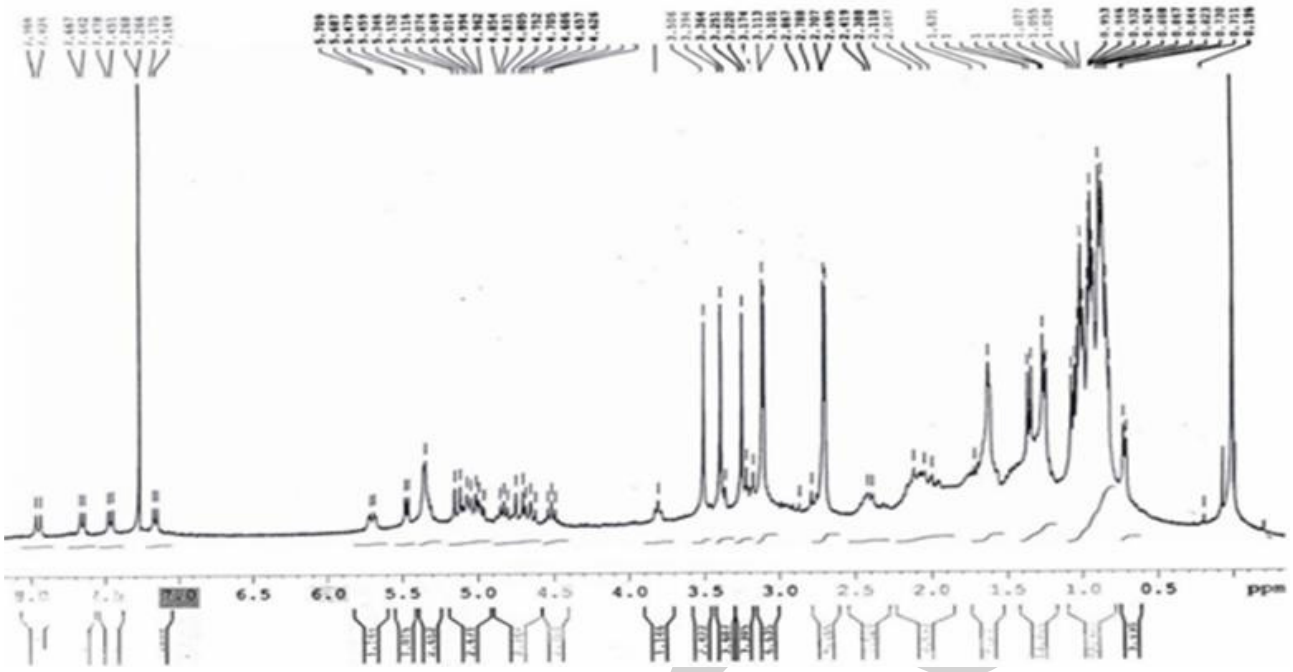

Fig. 2: ^{1}H NMR spectrum of purified Cyclosporine A

at a concentration of 250 μg disc^{-1}. The stock solution for the test was prepared by dissolving 0.25 g of each fraction in 1 ml of solvent (either in methanol, acetone or DMSO depending upon the solubility of extracts). Final concentration of 250 μg disc^{-1} of each stock solution was obtained by spreading 10 ml of stock solution on the paper disc. The active fractions were further purified by column chromatography over Sephadex LH 20. Active ethyl acetate fraction was chromatographed over Sephadex LH 20 with chloroform: methanol (1:1) as the eluent. The chromatographed fractions that were collected by column chromatography were pooled together on the basis of TLC profiles and were further purified wherever necessary, using Sephadex LH 20 columns. Sub-fractions were once again tested for the antifungal activity at a concentration of 250 μg disc^{-1}. Column chromatography and the testing for antifungal activity were repeated until the active principle was isolated, and further analyzed for it's chemical structure using IR, ^{1}H NMR and ^{13}C NMR spectra.

Results and Discussion

Three major fractions which were obtained from the acetone extract of *M. nivale*. The petroleum ether fraction was inactive against all the strains of fungi. Ethyl acetate fraction, however' showed significant activity against *A. niger, A. japonicus* and *A. fresenii* and dermatophytic forms like *C. neoformans*, *Candida* sp., *M. gypsum*, *T. tonsurans*, *T. violaceum*, *T. mentagrophytes*, *T. rubrum* and species of *Fusarium* (Table 1). However, an aqueous fraction showed mild to significant activity against the dermatophytes and *Aspergillus* species. Ethyl acetate fraction further yielded four sub-fractions. The antifungal activity was observed to be located in the sub-fractions F ·1-1 and F ·1-2. The maximum activity was shown by sub fraction F ·1-1, which further yielded three (F ·1-1-2 to F ·1-1- 4) active fractions exhibiting the highest activity against all the tested fungal strains. The volume of each fraction was ~ 5ml and total volume of active fraction was ~ 15 ml. This active fraction of ethyl acetate is in the form of amorphous white powder with Rf 0.50 (chloroform – methanol = 96:4) was obtained by TLC. Approximately 68 mg of the pure compound was extracted from ~ 7.90 g dry weight *M. nivale* mycelial mass.

Infrared (IR) spectrum of this compound exhibited absorption at 3437 cm^{-1} indicating the presence of hydroxyl group. The absorption at 2960 and 2874 cm^{-1} are characteristic of C-H stretch, indicating the presence of methyl group. Absorption peak located at 1689 cm^{-1} was indicative of C-N stretching vibration. It's ^{1}H NMR spectrum showed four doublets in the range of 7-8 ppm due to amide protons. About 23 protons corresponding to a- carbon proton of 11 amino acids and olefinic protons were indicated in the range of 4-6 ppm (Fig. 2). Seven peaks resulting from the N- methyl protons were evident in the range of 2.6-3.5 ppm. The ^{13}C NMR spectrum showed the presence of 61 carbon signals of which 8 were carbonyl carbons in the range 170.079-173.773.

The comparison of its IR, ^{1}H NMR and ^{13}C NMR as well as NMR experiments those reported earlier (Ruegger *et al*., 1976) helped in establishing the structure of the active fractions as Cyclosporine A.

A wide variety of carbon sources such as glucose (8% w/v) and sorbose (3.5% w/v) supported growth of Cyclosporine production, (Rehacek and De-Xiu 1991; Belaraman and Mathew 2006). However, the biosynthesis of naturally occuring Cyclosporine can be directed by externally supplying the precursor L-valine increased the specific production of Cyclosporine A. Cyclosporins are known to be a hydrophobic oligopeptide and exclusively

associated with lipoproteins in human serum having multiple bioactivities (Murthy *et al.*, 1999). During the present study the isolation and purification of cyclosporin A is reported from *M. nivale* a facultative marine fungus. The Cyclosporine A was confirmed by comparison of its IR and NMR values with those reported earlier (Ruegger *et al.*, 1976) helped in establishing the structure of the Cyclosporine A. Furthermore, these studies have also helped in the unambiguous assignment of almost all the carbon and proton signals. Initially Cyclosporine A was isolated as an antifungal compound. Recently antifungal activity of Cyclosporine A was described against phytopathogenic fungus *Sclerotinia sclerotiorum* (Rodriguez *et al.*, 2006). MIC of Cyclosporine A, against yeasts such as *C. neoformans* and *Candida* sp. was 12.5 - 25 mg $disc^{-1}$ (Table 2). *Cryptococcus neoformans* and *Candida* sp. is major medical importance and most frequently isolated. Cryptococcosis is one of the leading community acquired opportunistic mycoses (Hajjeh *et al.*, 1995). The serious diseases (*e.g.* meningitis and cryptococcemia) may occur in healthy hosts.

Dermatophytic fungi *Trichophyton* had reported to have link between sensitization to Asthma (Ward et al. 1989). In present study filamentous fungi *A. fresenii*, *A. niger*, *T. mentagrophytes*, and *M. gypsum* were reported to have MIC 25-50 mg $disc^{-1}$ as well as other filamentous fungi such as *A. japonicus*, *T. violaceum, T. tonsurans* and *Fusarium* sp., showed MIC of 12.5-25 μg $disc^{-1}$ (Fig. 1). However, antifungal activity of cyclosporin A against other fungal strains such as some species of the genera *Aspergillus, Curvularia, Trichophyton, Rhodotorula* and *Neurospora* have been linked to the inhibition of chitin synthesis (Cruz *et al.*, 2000). The antimicrobial activity of cyclosporin A has been related to the intracellar receptor cyclophilin A (Foor *et al.*, 1992). The complex cyclophilin A–Cyclosporine A might work as a potent inhibitor of different proteins, important in calcineurin metabolism (Breuder *et al.*, 1994). Calcineurin is required for appressorium morphogenesis and the regulation of hyphae growth (Viand *et al.*, 2002).

A fungus *M. nivale* isolated and purified from *P. coarctata* leaves during pre-monsoon and monsoon season. This fungus causes a disease to oat seeds and major leaves blight pathogen (Joshi and Miedaner, 2003). *Porteresia coarctata,* a host of *a M. nivale* occurs in beds and reported as common species in the process of mangrove formation along the estuaries in India (Jagtap *et al.*, 2006). However, genomic similarities of *P. coarctata* and *Oryza* species (*O. Schlechteri*) suggesting that the *P. coacrtata* is an *Oryza* species (Ge *et al.,* 1999). Furthermore, solid state fermentation studies using the commonly cultivated paddy species and *P. coarctata* plant would provide industrial applicable data. The solid state fermentation (SSF) has gained importance currently due to its several advantages over submerged fermentations (Pandey, 1992; Rodriguez-Couto *et al.*, 2006). Since SSF is being observed as a technique for producing higher yield of the desired products, various workers have shown interest for the production of high value microbial metabolites. Industrial production of Cyclosporine is carried out using the fungi in a liquid media as a submerged culture fermentation as well as solid state fermentation. The Cyclosporine A being hydrophobic molecules and has a much higher solubility in alcohols, such as methanol, ethanol, isopropanol as well as acetone (Canvanak and Sucker, 1986). The individual Cyclosporine products are separated from their closely related derivatives and metabolites using partition/ion exchange chromatography and HPLC. The retention time and peak area were compared with standard Cyclosporine A to get the level of cyclosporine A in the closely related derivatives. The industrial production of Cyclosporine A is mainly achieved using *Tolypocladium* genera. The Cyclosporine A obtained from *M. nivale* associated with *Porteresia* beds provided yet another source of Cyclosporine A from marine environment. The antifungal properties against *Aspergillus* species, yeasts and dermatophytes suggest that Cyclosporine A is potential candidate for life threatening diseases to human and animals.

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