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Antimicrobial susceptibility of anaerobic bacteria

In-vitro evaluation of marine derived fungi against *Cutibacterium acnes*

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ABSTRACT

Cutibacterium acnes (or *Propionibacterium acnes*) is the main target for the prevention and medical treatment of acne vulgaris. The aim of this study was to evaluate the *in vitro* anti-*C. acnes* and anti-*S. epidermidis* properties of some marine fungi isolated from different Indian marine environments. Seventy fungal isolates were obtained from samples collected from the west coasts and Andaman Island, India. Methanol extracts of 35 isolates were screened for their antibacterial properties and 5 out of the 35 isolates displayed significant inhibition as compared with tetracycline. DNA was successfully extracted from these five fungal isolates and phylogenetic analysis was performed. The methanol extracts possessed antibacterial activity against *C. acnes* and *S. epidermidis* with MIC values ranged from 0.8 mg/mL to 1 mg/mL. SEM analysis revealed that the extract induces deleterious morphological changes in the bacterial cell membrane. This study has identified some fungi extracts with significant antibacterial activity. The extracts may have potential for development as an antibacterial agent in the treatment of acne vulgaris.

1. Introduction

Acne vulgaris, commonly known as acne or pimples, is the most common skin disorder and it is characterized by inflammation of the sebaceous glands. It affects approximately 50 million people in the US, and many more throughout the world. Globally, more than 80% of the population suffer from acne at some stage in their life [1]. Acne can be extremely painful and causes lasting marks or scars as well as leads to psychosocial suffering [2]. Four factors including androgen-mediated stimulation of sebaceous gland activity, follicular hyper keratinization, colonization of the bacterium *C. acnes*, and inflammation, play important roles in the pathogenesis of acne [3]. In addition to *C. acnes*, as the main causative bacteria, *S. epidermidis* is also present in acne lesions [4]. Economically, it is estimated that US consumers spend more than 1.2 billion dollars each year for the treatment of acne [5]. Over a 10-year period the occurrence of skin colonization by antibiotic-resistant *C. acnes* in acne patients showed that the proportion of patients with strains resistant to one or more commonly used anti-acne antibiotics is increasing [6]. Given the trend of increased multidrug resistance, there is an urgent need to discover and development of promising new antimicrobials.

Marine natural products are an important source of drug leads [7–9]. Marine fungi are a valuable marine bioresource because of their abundant diversity and ability to produce chemically diverse secondary metabolites. In the last decade, thousands of novel bioactive compounds from marine fungi have been discovered that possess various pharmacological activities, including cytotoxic, anticancer, antiviral, antibacterial or antifungal activities [10–12]. Reports of antibacterial compounds from marine fungi have quickly increased since 2010, and marine fungi have been an important natural source of natural antibiotics [13]. For instance, an essential antibacterial indole-alkaloid was isolated from *Aspergillus flavus* OUCMDZ-2205, which exhibited strong activity against *Staphylococcus aureus* (MIC, 20.5 μM) [14]. Marine fungi *Eurotium cristatum* EN-220 was the source of Cristatumins A, which exhibited inhibitory activity against *S. aureus* at MIC, 64 μg/mL [15]. *Diaporthaceae* sp. PSU-SP2/4 gave Diaporthalasin, which displayed significant antibacterial activity against both *S. aureus* and methicillin-resistant *S. aureus* (MRSA) with equal MIC values of 2 μg/mL [16]. A xanthone derivative was isolated from *A. versicolor* MF359 and

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showed strong activity against *S. aureus* (MIC, 12.5 μg/mL) [17]. These finding indicate that marine fungi are a promising source for new antibacterial agents. In the present study, marine fungi from Indian marine environments were examined for antibacterial activity against inflammatory acne inducing bacteria *C. acnes* and *S. epidermidis*.

2. Material and methods

2.1. Chemicals used

All analytical grade chemicals were purchased from Fischer Scientific (Mumbai, India) and used without further purification. Potato dextrose agar (PDA), potato dextrose broth (PDB), malt extract, mycological peptone, brain heart infusion agar, brain heart infusion broth and Müller-Hinton agar were obtained from HiMedia (Mumbai, India). Streptomycin and penicillin were purchased from Sigma, (Mumbai, India). Diaion HP-20 resin was procured from Mitsubishi Chemical, (Japan).

2.2. Collection of samples

Various types of marine samples like algae, sea plants, sea sand and sear water were collected from different stations, including Gujarat, Goa and Andaman Islands in India. Sample collection was performed from different beaches of north and south Goa, which have very limited tourist activities like Arambol, Querim and Agonda beaches, and also from the marine estuary Chapora River, using a small boat. Samples were also collected from the Havelock beach in the Andaman Islands of India during Scuba diving. Marine algae and sediment samples were collected from a small pit of sea water near the sea in Dwarka, Gujarat. All the samples were collected from low tidal areas having high biodiversity and stored at 4 °C.

2.3. Isolation of marine fungi

Sea water samples were directly spread on the media plates (39 gm potato dextrose agar (PDA) in 1000 ml of naturally aged seawater, pH 7.5 ± 0.2, supplemented with 100 mg/L streptomycin and 50 mg/L penicillin as antibacterial agent) [18]. Algal samples were rinsed with sterile aged seawater several times until the sea sand was removed and then plated on media plates [19]. Likewise, plant material and dead animals were sterilized with 70% ethanol for 10 s to kill the residual epiphytes and washed with sterile seawater to remove ethanol, and dried with sterile tissue paper. Subsequently the samples were cut into pieces (1.0 × 0.1 cm size) using a sterilized blade and plated onto the media[20]. The sediment samples were diluted at 10 and 100 fold with autoclaved aged seawater and sea water was used directly as inoculum. 100 μl of each sample was spread onto PDA media plates. The experiment was performed in triplicates. All plates were incubated at room temperature (28 ± 2 °C) for 21 days. After 2 days of incubation media plates were examined daily for the presence of fungal growth. Distinct fungal colonies on the media plates were then transferred to new media plates with artificial sea water [21].

2.4. Cultivation of marine fungi for screening of antibiotic

All isolated fungal strains were cultured in 500 ml Erlenmeyer flasks containing 200 ml of potato dextrose broth (PDB, HiMedia) and malt extract broth (malt extract 17 gm/L, mycological peptone 3 gm/L) in artificial sea water, separately at 28 ± 2 °C for 20 days under shaking conditions at 140 rcf/g. After 20 days fermentation, whole culture broth (200 ml) was centrifuged to separate mycelium and supernatant. The supernatant was passed through a column of Diaion HP-20 resin (18 × 200 mm) previously equilibrated with water. The column was washed with distilled water until the eluent was no longer cloudy and then eluted with 50 ml methanol. Methanol was evaporated using a rotary evaporator (BUCHI Rota vapor R-200) and crude extract was collected and stored in the cold [22,23]. The fungal mycelia were also soaked in methanol for 24 h. The methanol layer was collected and evaporated to dryness to give the cell methanol extract, which was mixed with the above crude extract. The dry crude extracts were dissolved in sterile Milli-Q water [12] to prepare stock solutions of 5 mg/mL and used for antibacterial screening.

2.5. Antibacterial screening

2.5.1. Test organisms and bacterial inoculum

Two kinds of bacteria that cause acne, *Cutibacterium acnes* (MTCC-1951) and *Staphylococcus epidermidis* (MTCC-3615) were used as the test organisms. These were procured from Institute of Microbial Technology (IMTECH) Chandigarh, India.

A loopful of pure colonies of *S. epidermidis,* was inoculated into 20 ml nutrient broth and incubated at 37 °C for 4 h. *C. acnes* was inoculated into 20 ml of brain heart infusion media broth with 1% glucose for 72 h under anaerobic conditions. The turbidity of all actively growing bacterial suspension was adjusted to match the turbidity standard of 0.5 McFarland standard [(1.5×10^8 colony-forming unit, CFU/ml), prepared by mixing 0.5 ml of 1.75% (w/v) barium chloride dihydrate to 99.5 ml of 0.18 M (v/v)] sulphuric acid with continuous mixing. The bacterial suspension so prepared was used for testing their sensitivity to the samples under investigation [24].

2.5.2. In vitro antibacterial assay

An agar well-diffusion method described by Perez et al. [25] was used to determine the antibacterial activity. Mueller Hinton agar No. 2 (HiMedia, India) was prepared and cooled to 40 - 45 °C and the bacterial inoculum (1.5×10^8 CFU/mL, 0.5 McFarland) prepared above was then added aseptically to the molten agar and poured into sterile petri dishes to give a solid plate. Wells (6 mm diameter) were made in each of these plates using sterile cork borer. The wells were filled with 50 μl of test compound (5 mg/mL). The plates were first incubated at 4 °C for 15 min and then at 37 °C for 18–24 h under aerobic and anaerobic conditions, respectively. The antimicrobial spectrum of the extract was determined for the bacterial species in terms of zone sizes around each well. The diameters of zones of inhibition (ZOI) produced by the test were recorded and compared with those produced by positive control antibiotic, tetracycline (5 mg/mL, Sigma-Aldrich). For negative control pure solvent (sterile Milli-Q water) were used instead of the extract. The experiment was repeated two times in triplicates to minimize error and to check the reproducibility of results. The average values were recorded and % antimicrobial activity was calculated by applying the expression [26]:

$$\%\,\text{Inhibition} = 100 - \text{ZOI positive control} - \text{ZOI sample}/\text{ZOI positive control} \times 100$$

2.5.3. Determination of minimum inhibitory concentration (MIC)

The MIC of active extracts were determined by a microdilution assay in 96-well microtiter plates according to the dilution assay according to the EUCAST (http://www.eucast.org) [27]. The fresh cultures were prepared at 24 h and 72 h broth cultures of *S. epidermidis* and *C. acnes* and OD was set at 0.5 McFarland