Bioactive potential from Marine sponge *Callyspongia diffusa* associated *Psedumonas uorescens* BCPBMS-1 and *Penicillium citrinum*

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Abstract

Background: The exploration for marine sponge associated novel microbes, producing rich and highly potential therapeutic metabolites, could diversify the scopes in life sciences. Since this has remained mostly untouched, the research was carried out to explore the bioactive potential of a marine sponge, Callyspongia di usa associated microbes.

Materials and methods: The strains selected from the C. di usa were Pseudomonas fluorescens and Penicillium citrinum and their cell free extracts were tested for hemolytic activity on sheep blood agar media and antioxidant activity was assessed with lyophilized cell free extracts. Anticancer activity was performed by cytotoxicity assay against HEP-2 cell lines.

Results: Cell free extracts of both P. fluorescens and P. citrinum demonstrated α-hemolysis on sheep blood agar. The lyophilized culture filtrate of P. fluorescens BCPBMS-1 and P. citrinum exhibited concentration dependent antioxidant activity revealing a positive linear relationship and ca. 85% and 74% antioxidant activities were obtained respectively with 1.0 mg/ ml of each of the sample. In case of cytotoxicity assay, P. citrinum demonstrated maximum viability of 96.61% at 1.95 µg/ ml of lyophilized culture filtrate and minimum viability of 20.33% at 1000 µg/ ml.

Conclusion: The study proved that both P. fluorescens BCPBMS-1 and P. citrinum strains produce bioactive metabolites with hemolytic activity and antioxidant activity whereas P. citrinum could be a valuable resource for anticancer metabolites.

Signi cance | Marine microbes are potential resources for the treatment of metabolic diseases.

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Abbreviations: HEP-2; PDA, Potato dextrose agar; TAC, Total Antioxidant Capacity; MTT, 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; DMEM, Dulbecco's Modi ed Eagle Medium; FBS, fetal bovine serum; DMSO, Dimethyl sulfoxide; MCF-7, breast adenocarcinoma cell lines; NCI-H460, non-small lung cancer cell line; A375-15, melanoma cell lines; EPS, exopolysaccharides.

1. Introduction

Marine sponges are one of the rich sources of highly diverse microbial communities, including more than ten bacterial phyla (such as Proteobacteria, Actinobacteria, Nitrospira, Chloro exi, lanctomycetes, Cyanobacteria, Acidobacteria), major lineages of Archaea and a range of unicellular eukaryotes like diatoms and dino agellates. ese organisms as a whole are potentially useful because of their extensive metabolic diversity, including nitri ca-tion, photosynthesis, anaerobic metabolism and secondary metabolite production. However, the exact nature of the interactions between sponges and microbes is still an enigma to the scientists if the interaction is predation or parasitism or other types of symbiosis (Vasanthabharathi and Jayalakshmi, 2012).

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Novel marine natural products are isolated from marine bacteria, fungi, sponges, worms, shes and mostly from plants (Mayer & Hamann, 2002). ey are classi ed into six major chemical classes, namely, polyketides, terpenes, peptides, alkaloids, shikimates and sugars and have a wide variety of biological activities, such as antibacterial, anticoagulant, antimalarial, anti-in ammatory, antiprotozoal, antituberculotic and antiviral e ects (Abad, Bedoya, & Bermejo, 2008; Carballeira, 2008; Soltani, Saadatmand, Khavarinejad, & Nejadsattari, 2011).

Marine fungi are one of potential sources of secondary metabolites having various biological activities. *Penicillium brocae*, obtained from a tissue sample of the Fijian sponge *Zyzzya sp.*, produced three novel cytotoxic polyketides, Brocaenols A-C which showed cytotoxicity when tested against HCT-116 cell line. Bioactive extracts of α-Proteobacterial strains from the sponge surface as well as *Pseudomonas sp.* associated with primmorph exhibited antiangiogenic, antimicrobial, hemolytic and cytotoxic properties (omas, Kavlekar, & LokaBharathi, 2010).

Hemolytic power is considered as an important virulence factor for numerous bacterial pathogens. It is due to various factors such as pore-forming toxins, thiol-dependent cytolysins, enzymes like phospholipases, biosurfactants or to a concomitant action of these substances. Antioxidants are the molecules, which prevent cellular damage by reducing the oxidative stress and therefore have a bene cial e ect on human health. One of the major causes of mortality and morbidity world-wide is atherosclerosis, the accumulation of oxysterol, cholesterol, and peroxide lipids in arteries, generated by free radicals which lead to heart attack. Hence, there has been an increased interest in the application of antioxidants (Arora & Chandra, 2010; Rodrigues, Costa, Carvalho, & Epifanio, 2005).

2.0 Materials and methods

2.1 Isolation of bacteria and fungi from Callyspongia di usa

Isolation of bacteria

e sponge sample was collected from Mandapam Coast (Tamil Nadu, India), transferred to a sterile polyethylene bag and transported at 4 ºC to the laboratory for the isolation of associated micro-organisms. On reaching the laboratory, the invertebrate was brought to room temperature and cut aseptically into small pieces (2 × 2 cm) using a sterile scissors and washed twice with 2 ml of sterile seawater and vortexing for 20 s in order to remove adhering particles. Finally, the sample in sterile seawater was homogenized aseptically and the homogenate was serially diluted up to 10^{-6} dilutions and then spread plated on Zobell marine agar plates (Hi-Media, Mumbai) and incubated at room temperature for 24-48 hrs.

Isolation of fungi

1.0g of sponge sample was mixed in sterile water and was serially diluted up to 10^{-4}. 0.1 ml of the diluted sample was taken from 10^{-3} and 10^{-4} dilutions and was pour plated using 15-20 ml Potato dextrose agar (PDA) (Hi-Media, Mumbai) prepared in 50% sea water (to eliminate the bacterial contamination 8 ml of 1% Streptomycin was added to 1 L of the sterilized medium) and incubated at 30 °C for 5 days.

2.2 Identification of potential strain

e potential bacterial strain was identi ed by the conventional biochemical tests (Asha Devi, Rajendran, & Karthik Sundaram, 2011). Cell morphology was observed under a phase contrast microscope and con rmed through 16S rRNA gene sequencing.

e tree Topologies were evaluated by bootstrap analyses based on 1,000 replicates and phylogenetic trees were inferred using the neighbour-joining method and submitted to NCBI GenBank (accession number: 1428145 HQ907732). e sponge associated potential fungi were identi ed by following the method of omas et al. (Richards, Jones, Leonard, & Bass, 2012) and Hend et al. (Hend A. Alwathnani, 2012).

2.3 Hemolytic activity of potential strains

Hemolytic activity was determined using a blood agar plate. Blood agar base (Meat extract 10.0 g, Peptone 10.0g, Sodium chloride 5.0 g, Agar 15.0 g, pH 7.3±0.2 and distilled water 1000 ml) was prepared by autoclaving at 121 °C for 15 min. and allowed to cool at 45-50 °C and aseptically 5% (v/v) sterile de brinated sheep blood was added. Blood agar was poured into petri plates and wells were made.

50 µl of 24 h cell free extract of *P. uorescens* (5.0 x 10^6 cfu/ml) and 96 h cell free extract of *P. citrinum* (2.5 X10^6 cfu/ml) were inoculated on blood agar plate wells. Plates were examined for hemolysis a er incubation at 37 °C for 24 hrs. e plates were observed for zone of clearance as hemolysis was determined by a clear zone around the colony.

2.4 Antioxidant activity

e antioxidant activity was evaluated by the Phospho-molybdenum method according to the procedure of Vijayabaskar et al., 2012 (Vijayabaskar & Shiyamala, 2012). is assay is based on the reduction of Mo (VI) – Mo (V) by the extract and subsequent formation of a green phosphate / Mo(V) complex at acidic pH.

0.6 M sulfuric acid, 28 mM sodium sulfate and 4 mM ammonium molybdate were mixed together in 250ml distilled water and labeled as Total Antioxidant Capacity (TAC) reagent. Di erent concentration of lyophilized culture ltrate (0.2, 0.4, 0.6, 0.8, 1.0 mg/ml) of *P. uorescens* and *P. citrinum* were taken in separate test tubes. About 1 ml of TAC reagent was added to all tubes. Blank was prepared with distilled water replacing the TAC reagent. Absorbance was measured at 695 nm in a spectrophotometer where Gallic acid was used as standard. e total antioxidant activity was measured as follows:

$$\text{Percentage of total antioxidant activity} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

 https://doi.org/10.25163/microbbioacts.11002A2221300318

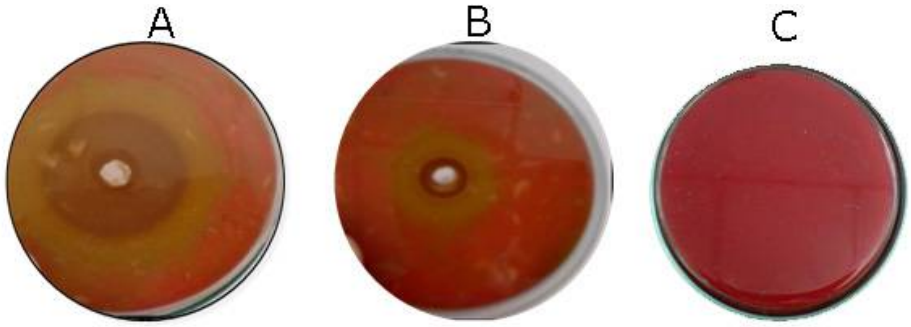

Figure 1 | **Assessment of hemolytic property. A) Hemolytic activity in culture filtrate of P. fluorescens, B) Hemolytic activity in culture filtrate of P. citrinum, C) Control (uninoculated)**

2.5 Cytotoxicity assay

Cytotoxic property of the bacterial strain was carried out by MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay against HEp-2 cell line. HEp-2 cells were grown in Dulbecco's Modi ed Eagle Medium (DMEM) which was supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml streptomycin. 100 µl of cell suspension was seeded into 96-well plates (5 × 103 cells/well) and incubated at 37 °C for 24 h. A er 24 h, lyophilized cell free extract of *P. uorescens* and *P. citrinum* at various concentrations (ranging from 1 mg/ml to 1.95 µg/ ml) were added and incubated at 37 °C + 5% CO_2 for 48 h. A er 48 h, media was removed from the wells carefully for MTT assay. Wells were washed with MEM (w/o) FCS for 2-3 times and 200 µl of MTT (5 mg/ml) was added and incubated again for 6-7 h. en 1ml of DMSO was added to each well and mixed by a pipette and le for 45 sec. e suspension was transferred into the cuvette of spectrophotometer and absorbance was taken at 595 nm where DMSO was used as blank. e % of cell viability was measured by the following formula:

Cell viability (%) = (Mean OD/Control OD) x 100

3.0 Results

3.1 Isolation and identification of potential strains

A er biochemical analysis, phase-contrast microscopy and 16s rRNA gene sequencing, highly potential bacterium *P. uorescens* and fungus *P. citrinum* were identi ed and selected for further characterization.

3.2 Hemolytic activity of potential strains

e present study showed alpha (α) type of hemolysis in culture ltrate of *P. uorescens* and *P.citrinum*. Alpha hemolysis refers to the partial lysis of red blood cells and hemoglobin. is resulted in a greenish-grey discoloration of the blood around the well (Figure 1).

3.3 Antioxidant activity

In the present study, lyophilized culture ltrate of *P. uorescens* and *P. citrinum* showed concentration dependent antioxidant activity and it was incereasing linearly with gradual increase in concentration and exhibited 85% and 74% antioxidant activity in

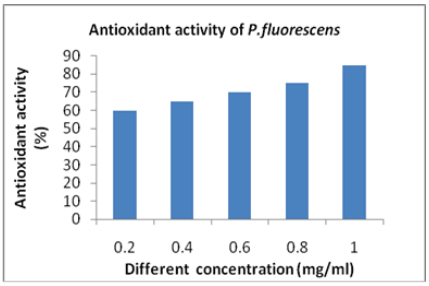

Figure 2 | **Antioxidant activity of P. fluorescens**

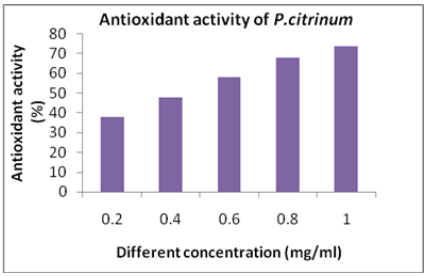

Figure 3 | **Antioxidant activity of P. citrinum**

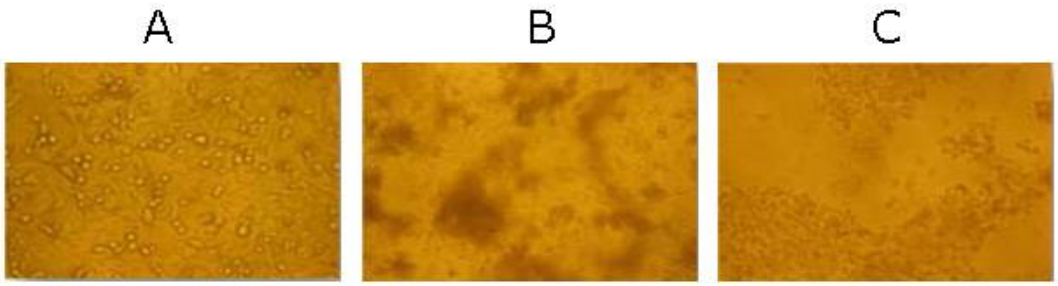

Figure 4 | **Cytotoxicity assay in HEp-2 cells for lyophilized extract of P. fluorescens BCPBMS-1. A) low toxicity, B) medium toxicity, C) high toxicity.**

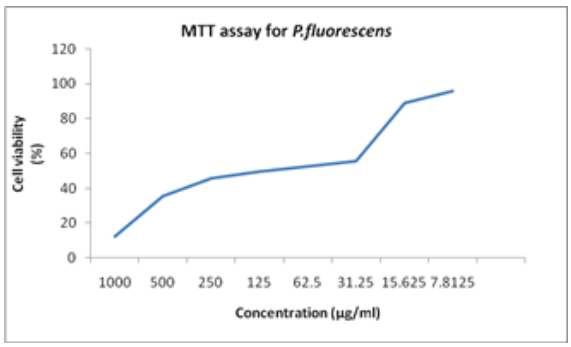

Figure 5 | **MTT assay for P. fluorescens**

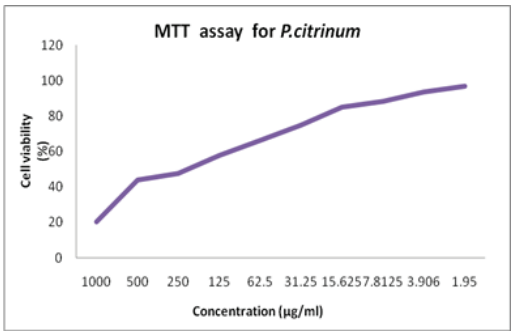

Figure 7 | **MTT assay for P. citrinum**

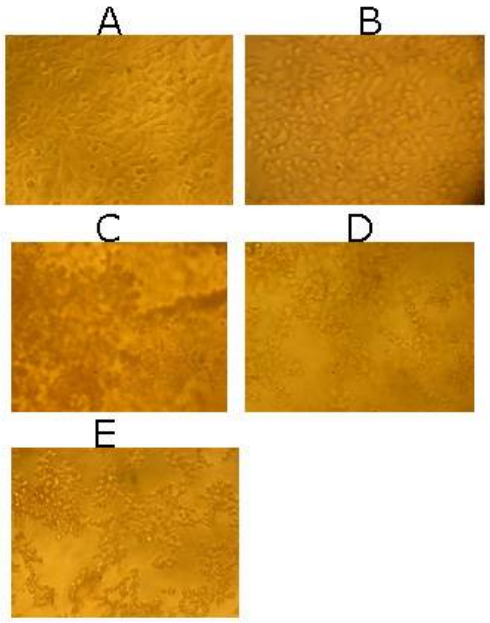

Figure 6 | **Cytotoxicity assay HEp-2 cells for lyophilized extract P. citrinum. A) Normal HEp-2 cell line B) Mild toxicity, C) Medium toxicity, D-E) High toxicity**

1 mg/ml of the sample, respectively. (Figure 2 and Figure 3).

3.4 Cytotoxicity assay

Toxicity was increased with increasing concentration of lyophilized cell free extract of *P. uorescens* ranged from 1 mg/ml to 7.8125 µg/ml. Maximum viability was observed at 7.815 µg/ml for the cell free extract of *P. uorescens* (95.68%) where at 1 mg/ml of cell free extract of *P. uorescens* viability count was 12.28%. In control (without lyophilized cell free extract of *P. uorescens*) viability was 100% (Figure 4 and Figure 5).

In case of *P. citrinum* toxicity was found to increase with increasing concentration of cell free extract of this fungus ranged from 1 mg/ml to 1.95 µg/ml. Maximum viability was observed at 1.95 µg/ml of cell free extract of *P. citrinum* (96.61%) and minimum at 1000 µg/ml which was 20.33%. Cell viability was 100% in control (only medium) (Fig 6 and Fig 7).

4. Discussion

e present study showed alpha (α) type of hemolysis in culture ltrate of *P. uorescens* BCPBMS-1 and *P. citrinum*. Most hemolysis-positive strains belonged to the genera *Pseudoalteromonas*, *Aeromonas spp.* and *Bacillus spp* (Romanenko, Uchino, Kalinovskaya, & Mikhailov, 2008). e extract of *Pseudomonas spp.* PB2 associated with a sponge, Suberites domuncula, exhibited anti-angiogenic, hemolytic, antimicrobial and cytotoxic activities (akur et al., 2005). Atagazli (Atagazli, Greenhill, Melrose, Pue, & Warner, 2010) observed hemolytic activity in culture ltrate of *P. citrinum* that yielded 80- 100% hemolysis in human erythrocytes. Hemolytic activity was observed in other Penicillium spp. as well (Taira, Marcondes, Mota, & Svidzinski, 2011). Bonassoli et al., (Bonassoli, Bertoli, & Svidzinski, 2005) reported hemolytic activity in *Candida arapsilosis*.

Antioxidant compounds scavenge free radicals such as peroxide, hydro peroxide or lipid peroxyl and thus reduce the level of oxidative stress slowing down or preventing the development of complications associated with oxidative stress related diseases. Many synthetic antioxidants have shown toxic and mutagenic e ects, which have shi ed attention towards naturally occurring antioxidants. In the present observation, lyophilized culture ltrate of *P. uorescens* and *P. citrinum* showed concentration dependent antioxidant activity which incereased linearly with gradual increase in concentration and exhibited 85% and 74% antioxidant activity in 1 mg/ ml of the sample, respectively.

Endophytic Paenibacillus polymyxa isolated from the root tissue of Stemona japonica, produced exopolysaccharides (EPS) which had strong scavenging activities on superoxide and hydroxyl radicals (Liu et al., 2009). Graphislactone-A, a phenolic metabolite isolated from the endophytic fungus Cephalosporium spp. IFB-E001, had free radical-scavenging and antioxidant activities in in vitro study (Song, Huang, Sun, Wang, & Tan, 2005). Guo et al. (Guo et al., 2010) reported that extracellular polysaccharides ETW1 and ETW2 produced by marine bacterium Edwardsiella tard, exhibited strong antioxidant activities. Antioxidant activity

was observed in intra-cellular and extra-cellular metabolites of marine Streptomyces species VITTK3 (enmozhi, Sindhura, & Kannabiran, 2010). Sun, et al., (Sun et al., 2009) isolated three di erent exopolysaccharides from marine fungus *Penicillium sp.* F23-2 and evaluated their antioxidant activity by assays in in vitro systems which revealed that those three polysaccharides possessed good antioxidant properties, especially scavenging abilities on superoxide radicals and hydroxyl radicals. Srinivasan et al. (Srinivasan et al., 2010) observed it in fungal extract of endophytic *Phyllosticta spp.* Sadananda et al. (Sadananda et al., 2011) reported total antioxidant capacity of the endophytic fungus A. niger and A. alternata. In the present observation also endorsed the same.

e cytotoxicity of lyophilized cell free extracts of sponge-associated bacteria and fungi against HEp-2 cell line indicated that the presence of potent cytotoxic and probably anticancer components of these extracts. Cytotoxicity was increased with increasing concentration of lyophilized cell free extract of *P. uorescens* and *P. citrinum* ranged from 1 mg/ml to 7.8125 µg/ml where highest cytotoxicity (87.72%) was observed in cell free extract of *P. uorescens* with a concentration of 1 mg/ml. Cytotoxicity against normal cell lines is needed to be assessed to further characterize these highly potent anticancer cell free extracts.

ere were some reports on *P. aeruginosa* and *Bacillus sp.* in producing some biologically active compounds against cancer cell lines (Ohba, Mizuki, & Uemori, 2009). e cytotoxic activity of the *Candida tropicalis, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Bacillus sp.,* crude extracts were determined against four established cancer cell lines; MCF-7, HepG2, HeLa and U937 cells, and Vero cell line as a representative of normal cell line (Kantachote et al., 2010). Alkaloid Lodopyridone from a marine *Saccharomonospora spp.* found to be cytotoxic (IC_{50} = 3.6 µM) to HCT-116 human colon cancer cells (Maloney et al., 2009). Sivonen et al. (Sivonen, Leikoski, Fewer, & Jokela, 2010) observed potent antitumor activitiy in ulithiacyclamide and patellamide-A belong to cyanobactins, produced by cyanobacteria. Phonnok et al. (Phonnok, Tanechpongtamb, & Wongsatayanon, 2010) reported cytotoxic activity of the microbial crude extracts against four established cancer cell lines, viz., MCF-7, HepG2, HeLa and U937 cells and Vero cell line. Yoghiapiscessa et al. (Yoghiapiscessa, Batubara, & Wahyudi, 2016) observed cytotoxic activity of (sponge) *Stylotella sp.* associated *Pseudoalteromonas avipulchra*. Marine derived fungus A. nomius (NC06) from sponge N. chaliniformis AR-01 showed the most selective cytotoxicity against WiDr cell line (Artasasta, Yanwirasti, Djamaan, & Handayani, 2017). Xiaoling et al., (Xiaoling et al., 2010) observed that mangrove associated endophytic fungi isolated from Zhuhai,China had cytotoxicity activity in KBV and KBV 200 cell lines. Almeida et al. (Kijjoa et al., 2010) observed anticancer activity in extract of *E.cristatum*, a fungi isolated from sponges. In vitro study proved its inhibitory activity against MCF-7 (breast adenocarcinoma), NCI-H460 (non-small lung cancer) and A375-15 (melanoma) cell lines.

5. Conclusions

e study proved that both *P. uorescens* BCPBMS-1 and *P. citrinum* possess good antioxidant activity as well as potent anticancer property. e active components responsible for these activities need to be evaluated. e data may contribute to a rational basis for the use of antioxidant rich marine *P. uorescens* and *P. citrinum*.

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Competing financial interest

e author(s) declare no competing nancial interests.

Author contributions

VV designed the whole research and nalized the manuscript and JS performed the experiments and dra ed the manuscript.

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