A Newly Isolated *Pseudomonas* sp., Epibiotic on the Seaweed, *Padina tetrastromatica*, off Southeastern Coast of India, Reveals Antibacterial Action

Aishwarya Ravisankar · Mary Elizabeth K. Gnanambal ·
Lakshmi R. Sundaram

Abstract Epibionts from the red (*Hypnea valentiae*) and brown seaweeds (*Padina tetrastromatica*) were rapidly isolated on Zobell agar medium. All the isolates from both the seaweeds (76 numbers) were tested against five human pathogens which were resistant to at least one of the commercially available antibiotics at a minimal concentration of 10 mg. The most antibiotic productive isolate (PT19) from *Padina tetrastromatica* was extracted and observed to inhibit *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* with zone sizes of 15 and 10 mm radius, respectively, at a concentration of 300 μg. Further, a direct bioautography was done and an inhibition was witnessed against the aforementioned pathogens even at 2 μg concentration around three spots (R_f values 0.6, 0.7, and 0.8). Preparative thin-layer chromatography yielded a yellow sticky compound (6 mg) which was identified as an alkaloid. The compound on reversed-phase high-pressure liquid chromatography analysis yielded two major and two minor peaks with retention times, 3.1, 4.2, 4.7, and 4.9 min, respectively. The antibacterial compound was recorded 96.6 % pure, and the producer strain was identified as *Pseudomonas* sp. To our knowledge, we are the first to isolate and identify *Pseudomonas* from *Padina tetrastromatica* producing antibacterial alkaloids. This study will pave way for exploring more bacterial load from the said algal groups for bioactivities.

Keywords *Padina tetrastromatica* · Macroalgae · Antibacterial · Alkaloid

Introduction

The macro-algal communities of *Hypnea valentiae* and *Padina tetrastromatica* are found in abundance in the southeastern coast of India, especially near Tuticorin areas. There have been many investigations focusing on the ecological [1–3] and biochemical aspects [4–6] of

A. Ravisankar · M. E. K. Gnanambal (✉)
Department of Biotechnology, Sri Ramachandra University, Porur, Chennai 600116, India
e-mail: drelizabethrajesh@sriramachandra.edu.in

L. R. Sundaram
Central Research Facility (CRF), Sri Ramachandra University, Porur, Chennai 600116, India

the said organisms; however, works focused toward finding out the epiphytic bacterial population and indicating that their protective roles in contributing toward seaweed defense mechanisms are very scarce. Marine structures are, in general, rapidly colonized by bacteria. Only certain biological surfaces resist colonization to variable degrees for more or less extended periods [7]. Epiphytic bacteria growing on the surfaces of the lamina of the seaweeds and other invertebrates live in a highly competitive environment, and this factor of course may be attributed to the production of antibiotics in these epibionts [8]. In fact, Lemos et al. [9] postulate that bacteria isolated from lone sources like seaweeds alone account to 20 % of antibiotic producers which are potentially active.

Despite this fact, both the aforementioned macroalgae have not been explored *hitherto* as potential sources for the isolation of potent antibacterial epibionts. We hypothesized that isolating epibionts from these sources could render us with potent antibacterial strains which could be novel because Tuticorin coastal areas is one of the most productive ecosystems with greatest biodiversity. Therefore, with this notion, the present investigation is carried out to isolate the bacteria associated with the red seaweed, *H. valentiae*, and brown seaweed, *Padina tetrastromatica*; to extract the active metabolites; and to arrive at a reversed-phase high-pressure liquid chromatography (RP-HPLC) spectrum of the active compound. Efforts are also taken to characterize the producer strain up to the genus levels.

Materials and Methods

Collection and Preservation of Samples

The red seaweed, *H. valentiae*, and the brown seaweed, *Padina tetrastromatica* (Fig. 1), were collected approximately 1.5 km off the harbor of Tuticorin coastal area (8°45′N, 78°13′ E) in the intertidal zone of a depth of 2–3 ft by opening sterilized falcon tubes deep inside water and collecting samples using sterilized forceps. Until such time, the samples brought to laboratory were preserved at 4 °C using dry ice. The salinity of water at the collection site was 35 ppt, and the temperature was 30 °C. Salinity was measured with a refractometer and temperature by a thermometer.

Isolation of Seaweed-Associated Bacteria

The seaweed samples were rinsed with filtered autoclaved sea water thrice to remove non-attached bacteria, and a small zone of (1 cm^2) was scrapped with a sterile swab, serially diluted, and spread on pre-poured Zobell marine agar (ZMA) plates per the method of

Fig. 1 Drying samples of *H. valentiae* and *Padina tetrastromatica*

Lemos et al. [9] with slight modifications. The plates were incubated for 48 h at 30 °C, and plates with perceptible differences in morphotypes and pigmentation were randomly selected and isolated by successful streaking and restreaking. The pure cultures were maintained at 4 °C on ZMA slants. A complete antibiogram of all the indicator strains was obtained, and we chose five strains that were at least resistant to one of the commercially available antibiotics at a minimal concentration of 10 mg.

Screening of Antibiotic Production by Marine Bacteria by Cross-Streak Method

Cross-streak method reported by Lemos et al. [9] with slight modifications was used for the preliminary screening of antagonistic activity. The marine cultures were streaked across the diameter of seawater based yeast extract peptone agar (peptone 0.5 %; yeast extract 0.3 %; bacteriological agar 1.5 % in filtered seawater) plates. The plates were incubated 24 h at 30 °C. Followed by this, the test strains were streaked at right angles across them and incubated for another 24 h at 30 °C. The antagonistic activity was scaled as a zone of clearance around the marine strain.

Isolation of Secondary Metabolites by Partitioning Experiments and Screening of Antibacterial Action

The potent strains conferring the highest inhibition were grown on 5 ml of Zobell marine broth (ZMB) and shaken at 290 rpm for 5 days at 37 °C. Volumes of 1 ml from each of the strains were vortexed at 290 rpm for 5 min, and the supernatant was added with equal volumes of ethyl acetate employing liquid–liquid extraction, mixed well, evaporated to dryness, and the metabolite yield was noted [10]. Metabolite concentrations of 50, 100, and 300 μg suspended in 75 μl dimethyl sulfoxide (DMSO) were added to predrilled wells of ZMA plates swabbed with pathogenic cultures. Control plates with 75 μl DMSO alone were also included, and all the tests were done in duplicates. The radius of zones of inhibition was noted per Nathan agar well diffusion technique [11].

Large-Scale Partitioning Experiments of the Active Crude Extracts

The most potent strain, PT_{19}, from *Padina tetrastromatica* was seeded to 200 ml of ZMB and allowed to grow for 5 days and was centrifuged. Partitioning protocols were adopted from Lippert et al. [12]. To the spent medium, equal volumes of ethyl acetate were added, shaken well, and left undisturbed for 30 min, and the less dense ethyl acetate layer at the top is selectively collected so as to obtain the medium-polar metabolites and evaporated to dryness thereafter. The metabolites were poured in pre-weighed Eppendorf, extract yield noted, and used for all further experiments. The most potent strain was again subjected to agar well diffusion method as stated above to check the reproducibility of preliminary results.

Thin-Layer Chromatography

The partition which showed the maximum activity was thin-layer chromatographed on commercially available thin-layer chromatography (TLC) plates (Merck, India) with pore size 60 Å with particle size 5–17 μm ($Silica_{60}$ coated on aluminum sheets) using solvents systems such as petroleum ether/ethyl acetate (5:5) and hexanes/ethyl acetate (5:5). The TLC plates were viewed under visible light and UV radiation [both short (254 nm) and long range

(366 nm)] on a Camag VU Cabinet, and photographs were taken for the purpose of analysis of the spot resolved at various R_f values.

Reversed-Phase High-Pressure Liquid Chromatography Analysis of the Active Ethyl Acetate Partition of PT$_{19}$

The active partitions of PT$_{19}$ which showed prominent activity were submitted to analysis on a Shimadzu Automated HPLC (LC 2010A HT). A volume of 160 μg of the ethyl acetate partition of PT$_{19}$ in HPLC grade methanol was injected at volumes of 20 μl to a C-18 column (4.6×250mm) of 5-μ-sized pores. The mobile phase used was acetonitrile/water (80:20) at a flow rate of 1 ml/min. Peaks were detected at 220 nm using UV detector, and the peak percentage and area were noted.

Direct Bioautography

The TLC plates containing resolved spot of different concentrations 2, 10, 25, 50, and 100 μg of the ethyl acetate extracts of the strain PT$_{19}$ were immersed with the soft agar (0.5 %) medium seeded 2.5 ml of 24 h broth culture containing test organism (OD values of 1) and 2 % aqueous solution of 2, 3, 5-triphenyl tetrazolium chloride and incubated at 30 °C for 16 h. A clear zone around a spot against pink background (live cells) indicated the presence of antibacterial agent [13].

Preparative Thin-Layer Chromatography of the Active Ethyl Acetate Partition and Activity Check

The ethyl acetate partition of PT$_{19}$ weighing 10 mg was subjected to preparative thin-layer chromatography (pTLC) (ethyl acetate/petroleum ether 1:1). The spot showing the activity with R_f values 0.6, 0.7, and 0.8 was scrapped off from the plate and was re-suspended in ethyl acetate, removed off silica fine particulates, supernatants containing the active components evaporated to dryness, and the yield noted. The active components were again subjected to activity testing by agar well diffusion method against *Klebsiella pneumoniae* at a concentration of 30 μg/50 μl DMSO. The active components were observed to have the same R_f values like those previously reported active ethyl acetate partitions of PT$_{19}$.

Phytochemical Analysis, TLC, and RP-HPLC of the Active Components

Using standard protocols [14], we attempted to analyze the phytochemical constituents (like alkaloids, glycosides, quinones, saponins, tannins, flavonoids, steroids, and sugars) of the active compound. Thereafter, the active components were again subjected to RP-HPLC for the sake of purity check and comparison as against the semi-purified partitions. The column conditions for RP-HPLC program of the pTLC purified spot was the same as followed previously. However, the mobile phase used was acetonitrile/water (70:30), and peaks were detected at 235 nm using UV detector and the peak percentage and area were noted.

Identification of the Potent Strain, PT$_{19}$

The potent antibacterial isolate from the seaweed, *Padina tetrastromatica*, PT$_{19}$ was subjected to morphological and biochemical tests to identify the genus level status of the

organism. Colony morphology which includes the configuration, margin, elevation, surface, pigmentation, opacity, cell size, shape, arrangement, and sporulation of the colony was identified. Growth at various temperatures, pH, and NaCl concentrations was also determined. Routinely employed biochemical tests like growth on various selective media; utilization of specific substrates; H_2S and gas production; hydrolysis of casein, gelatin, starch, esculin, arginine, and tween (20, 40, 60, 80); nitrate reduction; acid production from rhamnose, arabinose, mannose, xylose, trehalose, and sucrose; etc were also performed, and the genus level identification of the potent isolate PT_{19} was made.

Results

Isolation of Seaweed-Associated Bacteria and Screening for Antibiotic Production

The total heterotrophic epibiotic load of the seaweeds, *H. valentiae* and *Padina tetrastromatica*, analyzed was observed to be 35 ± 2 and 41 ± 3.4 CFU/cm^2 both at 10^4 dilutions, respectively (Figs. 2 and 3a, b). In the present investigation, we isolated more than 35 bacterial colonies from *H. valentiae* and 40 from *Padina tetrastromatica*. We performed Gram's staining for all the isolates and noted the morphology of individual cells (data not shown here).

For both the seaweeds, the percentage of G$^+$ bacteria (84.6 ± 3 % for *Hypnea* sp. and 60 ± 2.5 % for *Padina* sp.) outnumbered than those of G$^-$ ones. Percentage of G$^-$ isolates was recorded as 15.4 and 40 for *Hypnea* sp. and *Padina* sp., respectively. A total of 65.38 % from *H. valentiae* were found to be non-pigmented, and 34.61 % were found to be pigmented. However, there were a very less population of pigmented bacteria isolated from *Padina* sp. (25 %) in comparison to *Hypnea* sp. (Fig. 4).

All the bacterial isolates from *H. valentiae* were tested against five human pathogens, and their antagonistic activity was verified using cross-streak method, among which two showed prominent antibacterial action as listed in Table 1. Isolates from *Hypnea* sp., VS_{23} inhibited methicillin-resistant (MR) *Staphylococcus aureus* (7 mm) and *S. aureus* (6 mm); similarly, VS_{24} inhibited *Escherichia coli*, *Pseudomonas aeruginosa*, *S. aureus*, and MR *S. aureus* with the clearance zones of 15, 15, 12, and 11 mm, respectively (Fig. 5a, b). Notwithstanding, a few other isolates from *Hypnea* like VS_2, VS_5, VS_9, VS_{14}, VS_{18}, and VS_{26} were also
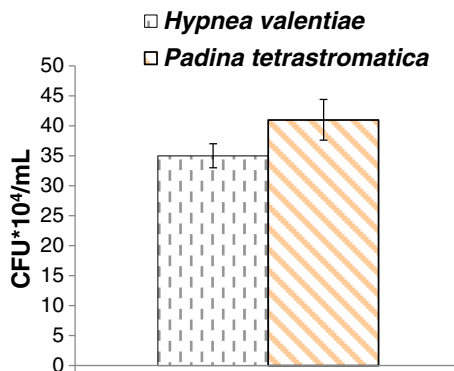

Fig. 2 Total heterotrophic epibacterial load of *H. valentiae* and *Padina tetrastromatica*
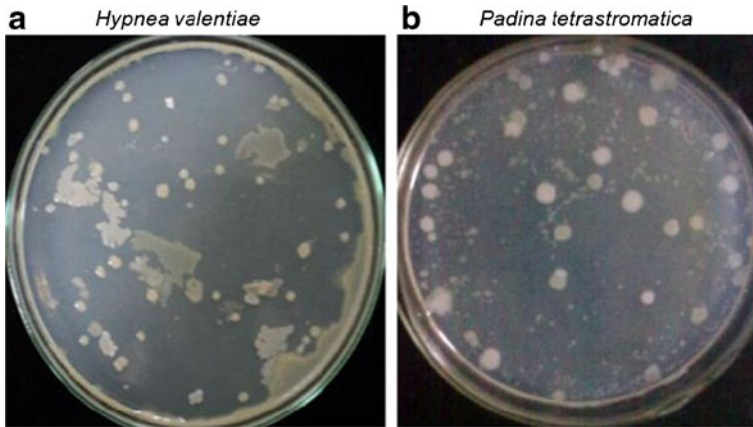
Fig. 3 **a, b** Total heterotrophic epibiotic population of the seaweeds 10^{-4} dilutions

able to inhibit a few pathogens with maximal inhibitory zones of merely 4 mm. Similarly, the isolate, PT_{19}, from *Padina* sp. was able to inhibit *K. pneumoniae* and *Pseudomonas aeruginosa* with zone sizes of 15 and 10 mm, respectively (Fig. 6) as against their counterparts. A few other isolates like PT_2, PT_9, PT_{12}, PT_{14}, and PT_{16} (Table 2) were, in addition, able to present inhibition to selective pathogens of varying degrees, however, could not be more antagonistic than PT_{19}. Out of the G^+ rods isolated from *Hypnea* sp. and *Padina* sp., 38 and 58 % were producers which were in higher number pronouncing antibacterial action than the G^- ones as figured in Fig. 7. For the case of *Hypnea* sp., the pigmented isolates dominated as potent producers (88.08 %), and for *Padina* sp., it was the non-pigmented population (53.33 %) that offered activity.
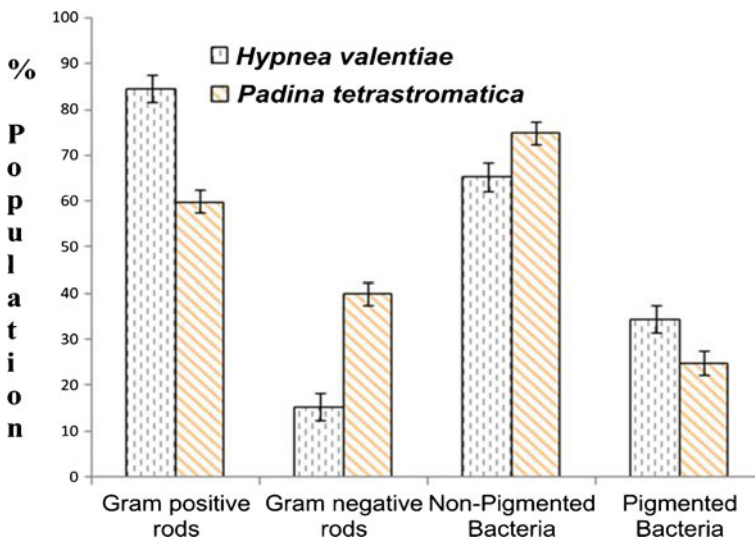

Fig. 4 Percentage of Gram-positive/Gram-negative rods/cocci and pigmented, non-pigmented isolated from *H. valentiae* and *Padina tetrastromatica*

Table 1 Antagonistic activity of epiphytic bacteria from *H. valentiae* by cross-streaking method

Epiphytic isolates of *H. valentiae*	Indicator strains				
	S. aureus	MR *S. aureus*	*E. coli*	*K. pneumoniae*	*Pseudomonas aeruginosa*
	Radius of the zones of inhibition (mm)				
VS_1	0	0	0	0	0
VS_2	0	0	2	3	0
VS_3	0	0	0	0	0
VS_4	0	0	0	2	0
VS_5	3	2	0	0	0
VS_6	0	0	0	0	0
VS_7	0	0	1	0	2
VS_8	0	0	0	0	2
VS_9	1	0	4	0	0
VS_{10}	0	0	0	0	0
VS_{11}	0	0	0	0	0
VS_{12}	0	0	0	0	0
VS_{13}	0	2	0	1	0
VS_{14}	0	0	2	3	0
VS_{15}	0	0	0	0	0
VS_{16}	0	0	0	0	0
VS_{17}	0	0	0	0	0
VS_{18}	0	3	0	2	0
VS_{19}	0	0	0	0	0
VS_{20}	0	0	0	0	0
VS_{21}	0	0	0	0	0
VS_{22}	0	0	0	0	0
VS_{23}	6	7	0	0	0
VS_{24}	12	11	15	0	15
VS_{25}	0	0	0	0	0
VS_{26}	2	0	1	1	0

Isolation of Secondary Metabolites by Partitioning Experiments and Screening of Antibacterial Action

Supported by our results from cross-streaking experiments, only VS_{23} and VS_{24} from *Hypnea* sp. and PT_{19} from *Padina* sp. were chosen for broth culture experiments and subsequent partitioning of the metabolites. None of the aqueous extracts was able to inhibit the pathogens, but for the ethyl acetate counterparts. The ethyl acetate partitioned metabolites at concentrations of 50, 100, and 300 μg of all the potent producers (VS_{23}, VS_{24} from *Hypnea* sp. and PT_{19} from *Padina* sp.) were dissolved in 50 μl of DMSO used for agar well diffusion assay for testing antibacterial activity. Pronounced inhibition was conferred by PT_{19} from *Padina* sp. against *K. pneumoniae* and *Pseudomonas aeruginosa* with zones of clearance of 15 mm diameter at 300 μg (Table 3) which could be much lesser in concentration than the commercially used antibiotics.
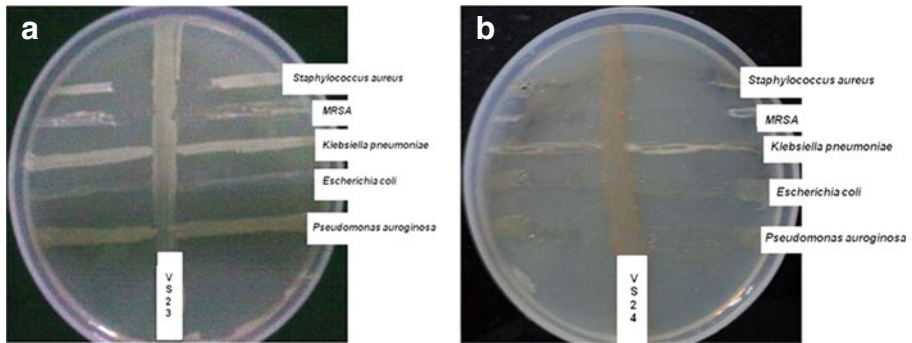
Fig. 5 Antagonistic activity of epiphytic bacteria **a** VS_{23} and **b** VS_{24} from *H. valentiae* by cross-streaking method

The isolate VS_{24} of *Hypnea* sp. elicited inhibitory activity against five pathogens tested with the maximum zones of 14, 11, 10, and 8 mm against *Pseudomonas aeruginosa*, *S. aureus*, MR *S. aureus*, and *E. coli*, respectively, at 300 μg concentration. VS_{23} of *Hypnea* sp. pronounced inhibitions of 15 and 13 mm against MR *S. aureus* and *S. aureus*, respectively (Table 3), at concentration of 300 μg. Based on these results figured as Fig. 8a–c, the producer conferring the highest zone of inhibition, PT_{19} of *Padina* sp., alone was chosen for mass culture experiments and subsequent extraction of metabolites for further analyses.

Thin-Layer Chromatography

The ethyl acetate partitions of *Padina* sp. epiphyte, PT_{19}, yielded a yellow-colored dry metabolite weighing 16 mg, which could be a mixture of compounds and hence purification required. The ethyl acetate partition was chromatographed in petroleum ether/ethyl acetate at ratios 1:1; resolved as various spot with R_f values 0.1, 0.6, 0.7, and 0.8 as figured in Fig. 9; and could be visualized only under long UV (366 nm) and UV short (254 nm) and not with white light.
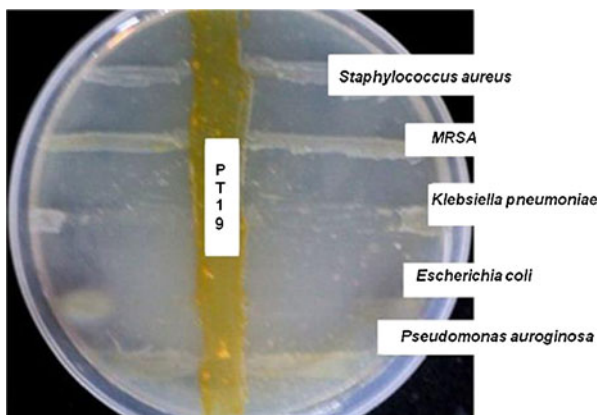

Fig. 6 Antagonistic activity of epiphytic bacteria of *Padina tetrastromatica* by cross-streaking method

Table 2 Antagonistic activity of epiphytic bacteria from *Padina tetrastromatica* by cross-streaking method

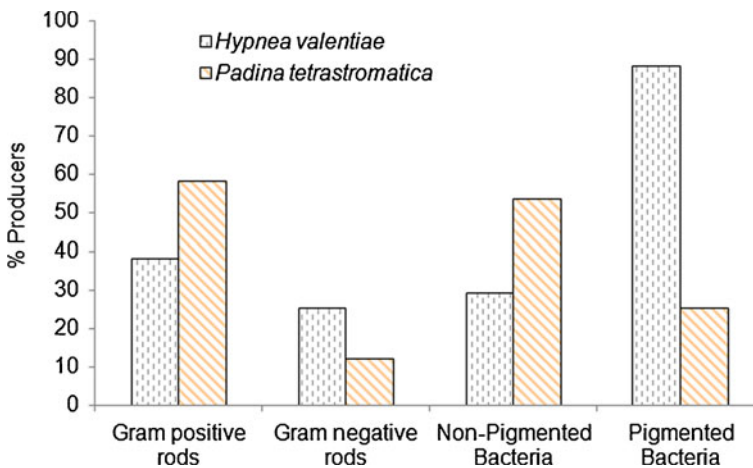| Epiphytic isolates of *Padina tetrastromatica*	Indicator strains				
	S. aureus	MR *S. aureus*	*E. coli*	*K. pneumoniae*	*Pseudomonas aeruginosa*
	Radius of the zones of inhibition (mm)				
PT_1	0	0	0	0	0
PT_2	3	2	0	0	0
PT_3	0	0	0	0	0
PT_4	0	0	0	0	0
PT_5	0	0	2	1	0
PT_6	0	0	0	0	0
PT_7	0	0	0	0	0
PT_8	0	0	2	0	1
PT_9	2	2	0	0	0
PT_{10}	0	0	0	0	0
PT_{11}	0	0	0	0	0
PT_{12}	1	3	0	0	3
PT_{13}	0	0	0	0	0
PT_{14}	0	0	1	4	0
PT_{15}	0	0	0	0	0
PT_{16}	2	3	0	2	0
PT_{17}	0	0	0	0	0
PT_{18}	0	0	0	0	0
PT_{19}	0	0	0	15	10
PT_{20}	0	0	0	0	0

Fig. 7 Percentage of producers from *H. valentiae* and *Padina tetrastromatica*

Table 3 Antibacterial action of the ethyl acetate partitions of the tested producer strains against selected human pathogens

Test strains	Diameter of zones of inhibition (mm)			
	Concentration (μg)	Producers		
		VS_{23}	VS_{24}	PT_{19}
S. aureus	50	0	0	0
	100	11	7	0
	300	13	11	0
MR S. aureus	50	3	4	0
	100	8	6	0
	300	15	10	0
E. coli	50	0	1	0
	100	0	5	0
	300	0	8	0
K. pneumoniae	50	0	0	1
	100	0	0	6
	300	0	0	15
Pseudomonas aeruginosa	50	0	3	6
	100	0	9	10
	300	0	14	15

Reversed-Phase High-Pressure Liquid Chromatography Analysis of the Active Ethyl Acetate Partition of PT_{19}

RP-HPLC spectrum of the ethyl acetate partitions of PT_{19} provided a profile with various peaks with different retention times. The major peak had a peak area of 43.88 % with retention times of 3.46 min followed by minor peaks of 21.6, 16.7, and 14.9 % with retention times of 3.8, 2.5, and 3.7 min, respectively, at 220 nm. We have also witnessed various minor peaks of not more than 2 % peak area (Fig. 10). However, the presence of numerous peaks required another RP-HPLC spectrum for the active compound. With the spectrum, it was possible to identify the medium polar nature of the compound, and the major peak could be further separated if purification strategies are followed.

Direct Bioautography

Simultaneously, a bioautography was done on resolved spot with various concentrations of spotted extracts (2, 10, 25, 50, and 100 μg) for pathogens K. pneumoniae and Pseudomonas aeruginosa. Pronounced zones of inhibition were detected around the resolved spot (R_f values 0.6, 0.7, and 0.8) even at a concentration of 2 μg (Fig. 11); however, as the concentration increased, the clearance of the zones was pronounced with apparently no change in the zone sizes.

Preparative Thin-Layer Chromatography of the Active Ethyl Acetate Partition and Activity Check

The ethyl acetate partition of PT_{19} weighing 10 mg was subjected to pTLC (ethyl acetate/petroleum ether 1:1). The active spot showing the activity with R_f values 0.6, 0.7,
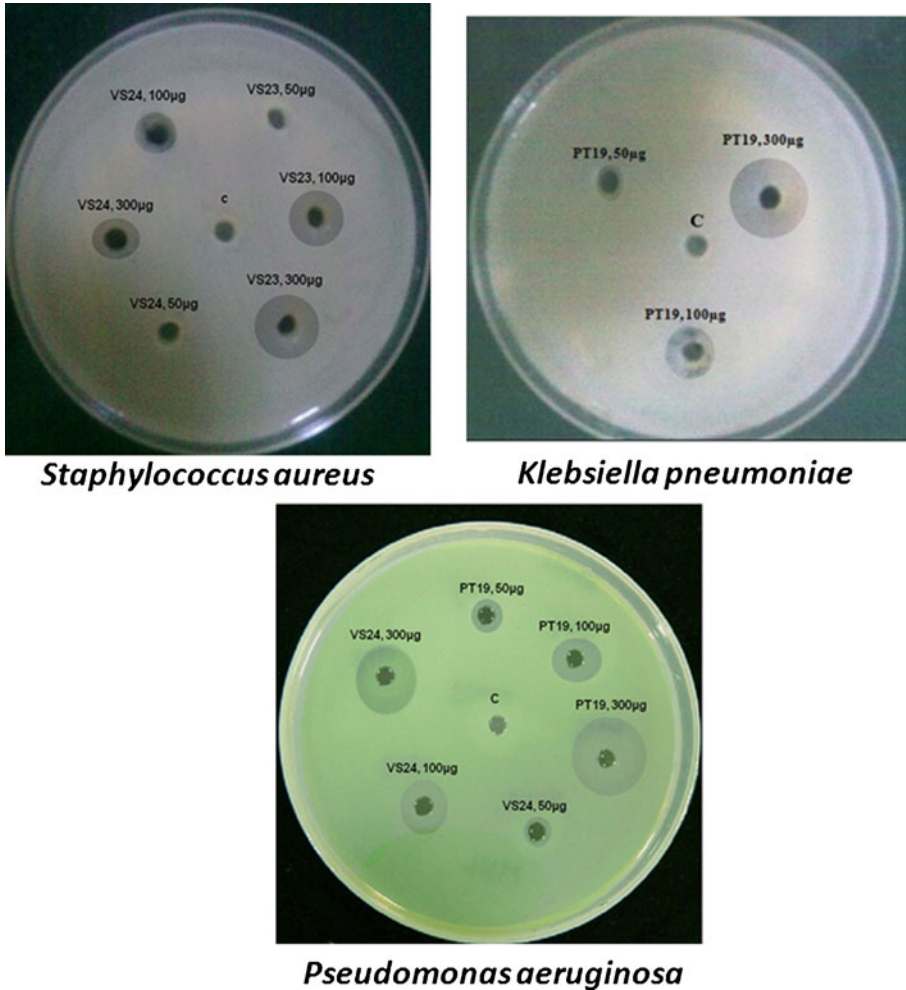
Fig. 8 a–c Antibacterial action of the ethyl acetate partitions of the tested producer strains against human pathogens

and 0.8 scrapped off from the plate as yellow sticky material was found to weigh 6 mg. A volume of 30 μg/50 μl DMSO of this substance was found to produce a zone of 16 mm diameter against *K. pneumoniae* (Fig. 12). The same compounds were again subjected to TLC analysis to check the purity of the active compound which was run with petroleum ether/ethyl acetate (1:1). The TLC resolved to three spots with R_f values 0.6, 0.7, and 0.8 as expected when compared with the TLC of the crude samples (Fig. 13).

Phytochemical Analysis, TLC, and RP-HPLC of the Active Components

Based on the morphological, biochemical, and physiological characteristics, the isolate PT_{19} from *Padina tetrastromatica* has been identified as *Pseudomonas* sp. Phytochemical screening indicated the presence of alkaloids which was confirmed by the positive results of Dragendroff's, Meyer's, and Wagner's reactions. The active spot upon RP-HPLC analysis
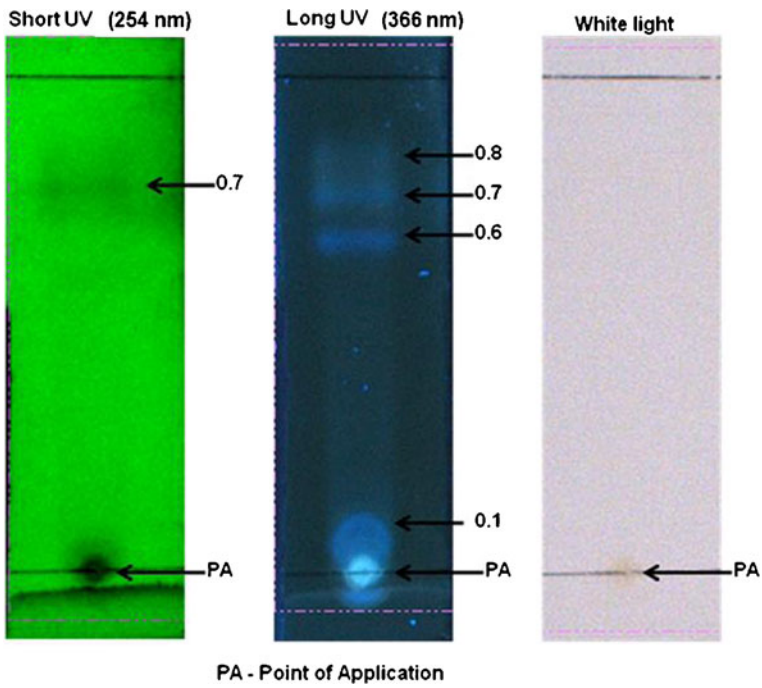
Fig. 9 TLC of active ethyl acetate partitions (petroleum ether/ethyl acetate 1:1) of PT_{19} in short UV, long UV, and visible light

yielded two major and two minor peaks with retention times 3.1, 4.2, 4.7, and 4.9 min, respectively (Fig. 14).
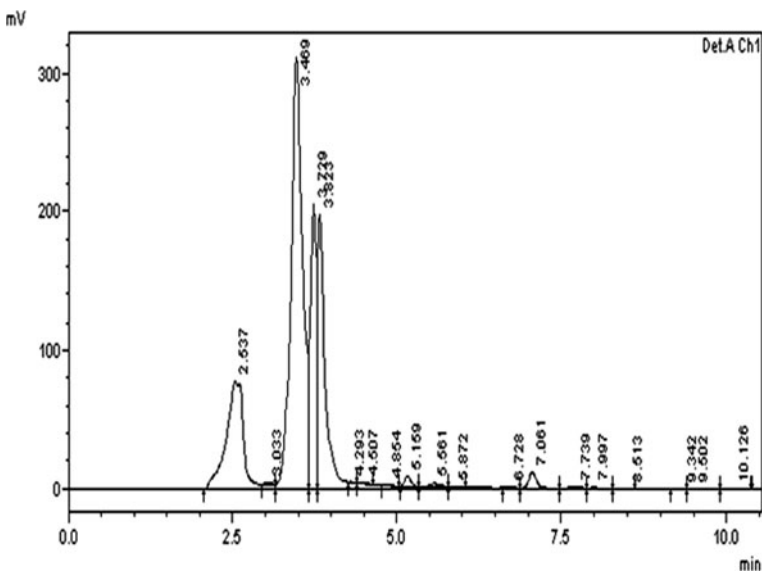

Fig. 10 Reversed-phase high-pressure liquid chromatography spectrum of the active ethyl acetate partition of PT_{19}
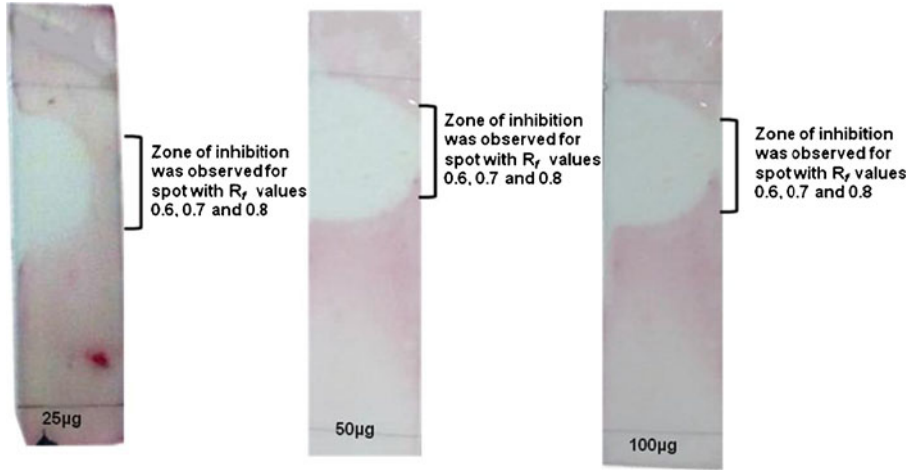
Fig. 11 Bioautography of metabolite from PT$_{19}$ (petroleum ether/ethyl acetate 1:1) against *K. pneumoniae*

Out of the two major peaks, one had a peak area of 63.2 % and the other with 29.8 %. The minor peaks had a peak area of not more than 4.4 % which may also indicate impurities. The RP-HPLC spectrum is in accordance with the TLC profile exhibiting three spots. Hence, it may be opinioned that the active spot as perceived by the spectral datum could be more than 96 % pure with minor impurities constituting to 4 %.

Discussion

Here we report for the first time the isolation of marine bacteria for extraction of some possible types of antibiotic substances from the epiphytes of *H. valentiae* and *Padina*
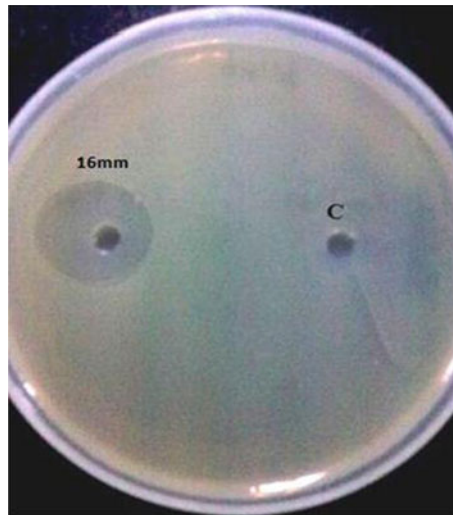

Fig. 12 Agar well diffusion of the active metabolite scrapped off from pTLC plates (30 μg) across *K. pneumoniae* lawns
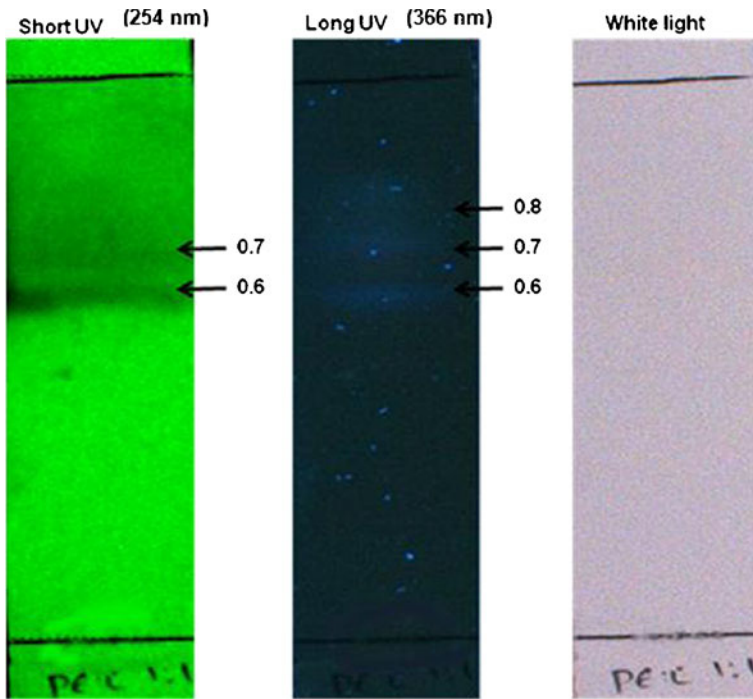
Fig. 13 TLC of the active spot (petroleum ether/ethyl acetate 1:1) of PT_{19} in short UV, long UV, and visible light

tetrastromatica and attempted to partially purify the compound exerting antibiotic capabilities against the human pathogenic bacteria, though works on the whole seaweed extracts of the said species are sparsely extant. Both the species form large proportion of the seaweed communities of the tropical countries, especially the southeast coast of India. Based on our studies, we found that these algal communities may harbor a few antibiotic producing epiphytes though often potent.
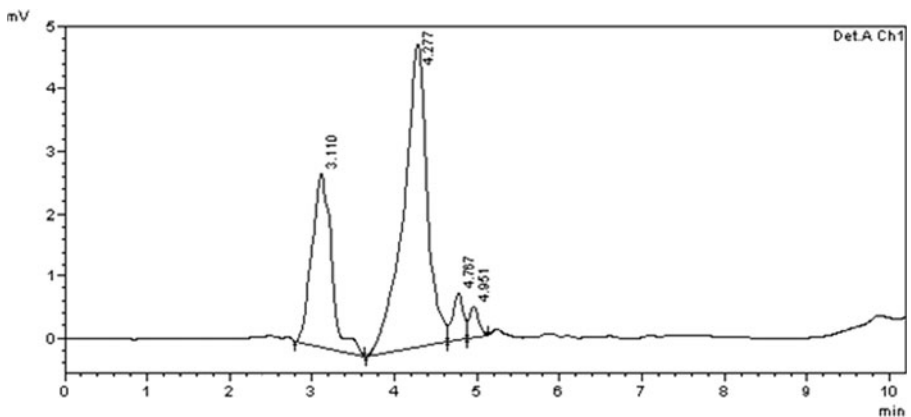

Fig. 14 RP-HPLC spectrum of the active spot of PT_{19}

It is usually argued that the total viable bacterial count of marine invertebrate ranged from 10^6 to a lowest of 10^3/cm^2 of marine invertebrates [15] and 10^9 in ocean-bottom sediments [16]. The same pattern is observed in our study that both the seaweed samples recorded observable viable counts per square centimeter at dilutions of 10^4. There is a relative abundance of G$^+$ bacteria from marine environs, especially from near-shore areas as specified by Jensen and Fenical [17] and indicated that 31 % of total viable bacterial load is G$^+$, which is factual with the present finding of more G$^+$ load in the tested algal communities. Percentage of G$^-$ isolates was recorded as 15.4 and 40 for *Hypnea* sp. and *Padina* sp., respectively. Our results contradicted with one of the previous works on *Sargassum* sp, where the G$^-$ predominated [18, 19]. It was not surprising that none of the isolates from both the macroalgae was cocci but rods because earlier workers have recovered only rods as the sole epiphytic load from seaweeds [9]. Pigmented bacterial population is, in general, postulated to be lower by 2–3 log counts of the total culturable marine bacteria recovered from marine invertebrates and algae [20–22] which is evident from the present study as well.

In the past, there have been umpteen efforts to establish the relationship of epibiotic bacteria attached to seaweed surfaces [8, 17, 23, 24]. Seaweeds always provide a comfortable space for its epibionts which often produce a battery of antibiotics [22, 25–27]. Also there are some supportive reports to documenting the protective role of these epiphytes, and in fact, many bioactive compounds from previously reported marine invertebrates are sourced only from the attached bacteria and not the invertebrates per se [28]. However, there is no report to substantiate the presence of symbiotic bacteria of *H. valentiae* and *Padina tetrastromatica* and evaluate the antagonism of these organisms against pathogenic bacteria. Hence, we report for the first time the antibiotic properties of few isolates from these two macroalgae. It is notable that a single isolate, VS$_{24}$, from *Hypnea* sp. inhibited four of the pathogens: *E. coli*, *Pseudomonas aeruginosa*, *S. aureus*, and MR *S. aureus* with the clearance zones of 15, 15, 12, and 11 mm, respectively. Similarly, the isolate, PT$_{19}$, from *Padina* sp. was able to inhibit *K. pneumoniae* and *Pseudomonas aeruginosa* with zone sizes of 15 and 10 mm, respectively This may be agreeable because inhibitory zone sizes of usually more than 5 mm have been reported for the epiphytic bacteria of macroalgae, *Fucus vesiculosus* and *Coralina officinalis* [8], *Laminaria saccharina* [29], *Codium fragile* [30], *Pockokiella variegata* [31], etc., indicating the potency of epibiotic isolates.

There is always a notion that marine G$^-$ rods are potent producers as postulated by Fenical [32]. Conversely, out of the G$^+$ rods isolated from *Hypnea* sp. and *Padina* sp., 38 and 58 % were producers which were in higher number pronouncing antibacterial action than the G$^-$ ones as figured in Fig. 7. It should not be misconstrued that a mainstream of antibiotic producers is G$^-$ rods; in fact, a bulk of producers had stemmed from the works with G$^+$ ones as corroborated in the present study. Baam et al. [33] also indicated that a majority of G$^+$ bacteria are antibiotic producers. It was constantly thought that only pigmented marine bacteria are chemically productive [34]. But there are contrasting observations noted with regard to pigmentation and antibiotic production in the current study. For the case of *Hypnea* sp., the pigmented isolates dominated as potent producers (88.08 %), and for *Padina* sp., it was the non-pigmented population (53.33 %) that offered activity. For the isolation of epiphytic bacteria from macroalgae, some authors have focused on pigmented colonies because their previous studies [35] and those of other authors [36, 37] demonstrated that antibiotic-producing marine bacteria were always pigmented. But our experiments demonstrate that even non-pigmented epibionts are chemically productive, however, with very meager activity. Hence, in accordance with previous works and our own experiment, non-

pigmented epiphytic bacteria from these two macroalgae may not be looked upon as a viable source for antibiotic production.

Partitioning experiments yielded more dense aqueous phases and less dense ethyl acetate ones. None of the aqueous extracts was able to inhibit the pathogens, but for the ethyl acetate counterparts because the antibacterial activity in ethyl acetate partitions of extracellular metabolites of marine bacteria is generally reported to be improved as against other organic phases [20, 27].

The concentration (300 μg) at which VS_{23} of *Hypnea* sp. pronounced inhibitions of 15 and 13 mm against MR *S. aureus* and *S. aureus*, respectively, is by far lesser than that used by [27] (2 mg) for Chinese seaweed epibacterial (ethyl acetate) extracts which was proposed to have presented inhibitory areas of only more than 5 mm. To our knowledge, there are no works indicative of reporting concentration of extracts of epibacterial isolates proving antibiotic; hence, a comparison could not be established.

In the experiments with autobiography, the spot with R_f value 0.1 did not show any activity suggesting that the medium polar metabolites of R_f values 0.6, 0.7, and 0.8 alone produced action for both the pathogens. Hence, the ethyl acetate partition of PT_{19}, as whole, is not potentially antibacterial, and of course, the action is only attributed to the medium polar metabolites of the extract. This indicates that the purified component as a whole is antibacterial as confirmed by agar well diffusion method. The active compounds strongly showed absorbance at various wavelengths across the UV range from 254 to 366 nm. Many of the marine epibacterial cellular components [38–41] produce UV absorbing components in order to withstand dilapidating effects of UV rays.

Phytochemical screening indicated the presence of alkaloids as indicated by many of the previous works. Marine alkaloids are often present in epibiotic organisms attached to invertebrates and are documented to demonstrate inhibitions against a variety of organisms at its vicinity [42, 43]. Marine alkaloids have ceaselessly being considered as potent antibacterial agents [44, 45]. Also there is copious information to quote the presence of alkaloid, particularly indole derivatives from epiphytic bacteria of marine invertebrates [46–48].

One interesting aspect of the isolated pseudomonad of the present study is that the creamish yellow pigmentation was developed only in the presence of sunlight and not once growth was witnessed in the incubator without sunlight source, except during primary isolation and the first streak. Development of pigmentation is always known to have a protective function against harmful UV radiations from entering into the cell membrane [49, 50]. This is true because Tuticorin is always associated with the high temperatures and long hours of intense sunlight [51] during most days of the year.

There are of course few literatures to support that yellow-colored alkaloids isolated from a variety of marine bacteria, inclusive of pseudomonads, are believed to be bioactive in nature [52, 53]. Thus, it may be clear that the presence of alkaloids in PT_{19} might have significantly contributed to the antibacterial action as explained in this study. It is notable that PT_{19} inhibited only G^- pathogenic isolates indicating that the antibacterial compound has specific inhibitory mechanisms which need to be understood.

The active spot upon RP-HPLC analysis yielded two major and two minor peaks. Examination on whether all the three peaks together or separately confer antibacterial action is currently performed to have an understanding on the exact active component. This study gives us a clue that a potent antibacterial alkaloid from a new epibiotic *Pseudomonas* sp. of *Padina* sp. could be effectively used as promising antibacterial drug in the future. In conclusion, it may be mentioned that the relatively less studied epibiotic communities on *Padina* sp. and *Hypnea* sp. may be looked upon as promising sources of drugs against both Gram-positive and Gram-negative bacterial pathogens.

References

1. Rangaiah, G. S., & Rao, M. W. (1983). *Plant Science, 92*, 473–482.
2. Subbaraju, L. V., Krishna, K. S., & Chaubey, A. K. (1991). *Journal of Coastal Research, 7*, 509–516.
3. Aisha, K., & Shameel, M. (1995). *Pakistan Journal of Botany, 27*, 41–48.
4. Kaladharan, P., & Velayudhan, T. S. (2005). *Seaweed Research and Utilisation, 27*, 35–37.
5. Rathinam, V. A., Jiang, Z., Waggoner, S. N., Sharma, S., Cole, L. E., & Waggoner, L. (2010). *Nature Immunology, 11*, 395–402.
6. Kamat, S. Y., Wahidulla, S., Souza, L. D., et al. (1992). *Botanica Marina, 35*, 161–164.
7. Fletcher, M., & Marshall, K. C. (1982). *Advances in Microbial Ecology, 6*, 199–236.
8. Burgess, J. G., Jordan, E. M., Bregu, M., Spragg, A., & Boyd, K. G. (1999). *Journal of Biotechnology, 70*, 27–32.
9. Lemos, M. L., Torenzo, A. E., & Barja, J. L. (1985). *Microbial Ecology, 11*, 149–163.
10. Chellaram, C., Gnanambal, K. M. E., & Edward, J. K. P. (2004). *International Journal of Marine Science, 33*, 369–372.
11. Nathan, P., Law, E. J., Murphy, D. F., et al. (1978). *Burns, 4*, 177–187.
12. Lippert, H., Brinkmeyer, R., Mülhaupt, T., et al. (2003). *Polar Biology, 26*, 591–600.
13. Meyers, E., & Smith, D. A. (1964). *Journal of Chromatography, 4*, 129–132.
14. Ayoola, G. A., Coker, H. A. B., Adesegun, S. A., et al. (2008). *Tropical Journal of Pharmacy Research, 7*, 1019–1024.
15. Austin, B. (1988). In: *Marine microbiology* (pp. 45–55). Melbourne: Cambridge University Press.
16. Fenical, W., & Jensen, P. R. (2006). *Nature Chemical Biology, 12*, 666–673.
17. Jensen, P. R., & Fenical, W. (1994). *Annual Review of Microbiology, 48*, 559–584.
18. Sieburth, J. M. (1968). *Advances in Microbial, 1*, 63–94.
19. Ramaiah, N., & Chandramohan, D. (1992). *Aquatic Botany, 44*, 71–81.
20. Vijayalakshmi, S., Ramaswamy, M. S., Murugesh, S., et al. (2008). *Annals of Microbiology, 58*, 605–609.
21. Gnanambal, K. M. E., Chellaram, C., & Patterson, J. (2005). *International Journal of Marine Science, 34*, 316–319.
22. Jayanth, K., Jeyasekaran, G., & Shakila, R. J. (2002). *International Journal of Marine Science, 31*, 45–51.
23. Bernan, V. S., Greenstein, M., & Maiese, W. M. (1997). *Advances in Applied Microbiology, 43*, 57–89.
24. Laycock, R. A. (1974). *Marine Biology, 25*, 223–231.
25. Spragg, A. M., Brega, M., Boyd, K. G., et al. (1998). *Letters in Applied Microbiology, 27*, 142–146.
26. Holmstrom, C., Rittschof, D., & Kjelleberg, S. (1992). *Applied and Environmental Microbiology, 58*, 2111–2115.
27. Zheng, L., Han, X., Chen, H., et al. (2005). *Annals of Microbiology, 55*, 119–124.
28. Proksch, P., Edrada, R. A., & Ebel, R. (2002). *Applied Microbiology, 59*, 125–134.
29. Wiese, J., Thiel, V., Nagel, K., et al. (2009). *Marine Biotechnology, 1*, 287–300.
30. Armstrong, E., Yan, L., Boyd, K. G. et al. (1995). *Hydrobiology, 461*, 37–40.
31. Imamura, N., Nishijima, M., Takadera, T., et al. (1997). *The Journal of Antibiotics, 50*, 8–12.
32. Fenical, W. (1993). *Chemical Reviews, 93*, 1673–1683.
33. Baam, R. B., Gandhi, N. M., & Freitas, Y. M. (1966). *Marine Research, 13*, 181–187.
34. Rosenfeld, W. D., & Zobell, C. E. (1947). *Journal of Bacteriology, l54*, 393–398.
35. Barja, J. L. (1979). *Marine Biotechnology, 2*, 225–230.
36. Anderson, R. J., Wolfe, M. S., & Faulkner, D. J. (1974). *Marine Biology, 27*, 281–285.
37. Gauthier, M. J. (1977). *International Journal of Systematic Bacteriology, 27*, 349–354.
38. Sunaryanto, R., Marwoto, B., Irawadi, T. T., et al. (2010). *Indonesian Journal of Chemistry, 10*, 226–232.
39. Kim, D. G., Moon, K., Kim, S. H., et al. (2000). *Journal of Natural Products, 12*, 1–9.
40. Fenical, W., Mercedo, I. E., Davo, A., et al. (2005). *Journal of Natural Products, 68*, 904–910.
41. Swaadoun, I., Hameed, K. M., & Moussauui, A. (1999). *Microbios, 99*, 173–179.
42. Gomez, L. J., Mercedo, I. E., Graciea, G. R., et al. (2010). *Oceanography Biological Markers Oceanography, 45*, 267–275.
43. Yu, L. L., Li, Z. Y., Peng, C. S., et al. (2009). *Helvetica Chimica Acta, 92*, 607–612.

44. Pelttari, E., Matikainen, J., & Hannu, E. (2002). *Zeitschrift für Naturforschung, 57*, 578–552.
45. Yin, S., Davis, R. A., Shelper, T., et al. (2011). *Organic & Biomolecular Chemistry, 9*, 6755–6760.
46. Roll, D. M., & Ireland, C. M. (1985). *Tetrahedron Letters, 26*, 4303–4310.
47. Moriarty, R. M., Roll, D. M., Ku, Y. Y., et al. (1987). *Tetrahedron Letters, 28*, 749–752.
48. Tanaka, J., Higa, T., Bernardinelli, G., et al. (1988). *Tetrahedron Letters, 29*, 6279–6284.
49. Porteau, P. J., Gerwick, W. H., Pichel, G. F., et al. (1993). *Experientia, 49*, 825–829.
50. Griffiths, M., Wrsistrom, G., Cohen-Bazire, R. Y., et al. (1955). *Natural, 176*, 1211–1214.
51. Sivaramasundaram, K., & Muthusubramanian, P. (2010). *Air Quality, Atmospheric and Health, 3*, 95–102.
52. Franks, A., Haywood, P., Holmström, C., et al. (2005). *Molecular, 10*, 1286–1291.
53. Pinkerton, D. M., Banwell, M. G., Garson, M. J., et al. (2010). *Chemistry & Biodiversity, 7*, 1311–1324.