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Research Article

Evaluation of Bioactive Secondary Metabolites from Ponyfish Associated Bacteria (*Photobacterium leiognathi)*

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ABSTRACT

The marine environment continues to surprise us by producing novel bioactive substances with a wide range of benefits for humans. Materials and Methods: Marine bioluminescent bacteria *Photobacterium leiognathi* was isolated from pony fish, *Secutorruconius* which was confirmed with microscopic and molecular characterization. The secondary metabolite of the isolated bacteria was extracted with dichloromethane. The chemical fingerprinting of the isolated metabolite was analyzed through TLC, FT-IR, and HPLC. The nature of the compound present in the metabolite was identified in the gas chromatography-mass spectrometry analysis (GC - MS). The isolated extract was investigated for its antibacterial property against 10 human pathogenic bacteria and also its antioxidant activity using different assays such as 1, 1-Diphenyl-2-picrylhydrazyl, Phosphomolybdenum, Metal chelating, Hydroxyl radical scavenging and hydrogen peroxide scavenging activity. Results: The Presence of functional groups including phenols, sugars, and amino acids in the extracts were identified by TLC. Totally, nine peaks were obtained for the crude extract through the FTIR spectrum range of 400 to 4000 cm^{-1} for the active sample. The DCM extract showed a broad spectrum of antibacterial activity against the six human bacterial pathogens. Secondary metabolites from the bioluminescent bacteria, *P. leiognathi,* have strong antioxidant properties. These results will be instrumental in developing novel products with biosensors and bio-imaging applications using *P. leiognathi.*

INTRODUCTION

Being one of the least investigated ecosystems with only one percent of the oceans being explored, the marine environment still contains many novel compounds with various applications including cosmetics, nutraceuticals, pharmaceuticals, feed formulation, food supplements, textiles, etc. One of the most understudied ecosystems on the planet is the marine environment. Many studies have identified marine natural products for a variety of purposes, including nutraceuticals, pharmaceuticals, feed formulation, textiles, and so on, although they represent only 1% of ocean exploration and abundance. On the other hand, still the marine environment, continues to surprise us by producing novel bioactive substances with a wide range of benefits for humans. Bioluminescence is one such characteristic that provides spectacular

advantages to the populace while also adding to the beauty of the ocean. Bioluminescence is produced by the extracellular biochemical compounds with chemiluminescence properties (Morin-Crini et al. 2019). Bioluminescence producing bacteria is attached to surface of several marine flora and fauna (Ramesh & Mohanraju 2017). The need for the production of bioluminescence molecules differs among species. Some species make biochemical substances to attract the opposite gender and to light up the deeper waters for food-seeking purposes.

Chemists have identified that the bioluminescence organisms could emit light at a visible range which is produced by naturally occurring enzymatic reactions. The photoprotein from some organisms emit light when combined with luciferin or luciferase enzyme, but not all photoproteins require them to emit light (Haddock et al. 2010). The majority of luminescent bacteria inhabits the ocean. Mainly, two genera of marine bacteria, *Vibrio* and *Photobacterium*, are the most abundant bioluminescent bacteria. They are found in seawater, intestinal tract, and on the surfaces of marine animals. However, the only terrestrial luminescent bacterial genus known so far is *Photorhabdus* (Engebrecht et al. 1983). *Photobacterium leiognathi* is a marine bacterium that can naturally emit light by secreting the photoprotein and reacting with that atmospheric oxygen. The bioluminescence proteins of the bacteria were highly sensitive to a wide variety of toxic substances including heavy metals such as Hg, Al, Zn and Cr (Kannahi & Sivasankari 2014).

The applications of bioluminescence are used for sensing and controlling hygienicity for many industries such as fish and milk industries. Moreover, bioluminescence-based assays can be applied for pollution mapping in ecosystems and sensing of pH, metal ions, transmembrane potential, drug molecules and other metabolites, gene assays and observation of protein–protein interactions (Kim et al. 2018; Sharifian et al. 2018). Bioluminescence research is also being conducted for use in the medical field. The bioluminescent bacterium has been used as an imaging component in the medical field (Nunes-Halldorson & Duran 2003; Menz et al. 2013). *P. leiognathi* was isolated from the marine ponyfish *Secutorruconius* and its secondary metabolite was extracted. Furthermore, the antibacterial and antioxidant properties of the secondary metabolite were evaluated using standard methods.

MATERIALS AND METHODS

Sample Collection and Bacterial Isolation

Live fish of *Secutorruconius* (ponyfish of the Leiognathidae family) were collected from Mudasalodai, Tamilnadu, India (Lat11°30'501" N and long 079° 49'669" E) and stored in an ice chest. The fishes were washed with sterile sea water then rinsed by double distilled water. The fish body surface was swabbed with sterile cotton swab for the isolation of luminescent bacteria. The upper layer of the skin of the fish was peeled off with the help of a sterile blade without touching the light organ and the tissue was homogenized with 1 ml of sterile seawater. The homogenate was serially diluted up to 10^5

and used for the isolation of luminescent bacteria. The swab and homogenate dilution (100µl) were spread on freshly prepared luminescent agar and Zobell marine agar plates. The plates were incubated at 22 °C for 24 hrs and monitored for the growth of luminescent bacteria. High-intensity light emitting luminescent bacterial colonies were sub-cultured for future works, the isolated pure colonies were subjected to morphological and molecular identification. The pure culture was also used for secondary metabolite production and purification. Further, the glycerol stock was also prepared and stored for future studies (Firudoz et al. 2020).

Morphological and Biochemical Characterization of Bacteria

The isolated pure luminescent bacterial colonies were subjected to morphological characterization by microscopy and colony characteristics. Initially, the cultures were subjected to the Gram staining technique to classify the bacteria according to the composition of the cell wall. Later, the cultures were grown on luminescent agar and are kept in dark to identify the luminescence of the bacteria, and the cultures were also grown in thiosulphate-citrate -bile salts-sucrose (TCBS) agar, a selective medium for identifying marine bacteria, especially *Vibrio* sp. The cultures were then subjected to a catalase test in which a loopful of liquid culture was added to a clean slide with 3% hydrogen peroxide and observed foran immediate effervescence. Another biochemical test is the starch hydrolysis test, in which cultures were inoculated in nutrient agar supplemented with starch at a final concentration of 2% and after incubation, the plates were flooded with iodine solution and clear zones around the culture as a result of starch hydrolysis (Sarkar et al. 2019).

Molecular Characterization of Bacteria

The use of molecular characterization helps in the specific identification of the isolate and thus was carried out accordingly. The genomic DNA of the isolate was extracted using a Bacterial Genomic DNA extraction kit according to the manufacturer's protocol (QIAGEN, QIAamp DNA Mini Kit) with some modifications. The isolated DNA was then amplified using the following PCR mix: 1 µl of bacterial universal 16S rRNA primers forward E9F (5-GAGTTTGATCCTGGCTCAG-3) (Farrelly et al. 1995) and 1µl of reverse primer U1510R (5-GGTTACCTTGTTACGACTT-3) (Reysenbach & Pac 1995), 2 µl of genomic DNA and 6µl of PCR grade water were added and the PCR amplification was done. Amplified sequence threads were submitted to the NCBI database and NCBI BLAST (http://www.ncbi.nlm.nih.gov/Blast) was carried out to distinguish the nearest neighbors of the isolates and then a phylogenetic tree was constructed using MEGA X software.

Extraction of Secondary Metabolites from Luminous Bacteria

A loop full of bacterial culture was inoculated in 250 ml conical flasks containing 150 ml Zobell marine broth and incubated at 28°C for 72 hours un-

der constant agitation of 220 RPM. Post-incubation, the cultures were taken and centrifuged at 6000 RPM for 20 minutes to collect the supernatant. To the collected supernatant, an equal volume of dichloromethane (DCM) was added and continuously agitated for 1 hour to obtain a homogeneous mixture. The organic phases were collected using a separating funnel and the solvent was evaporated using a rotary evaporator. The sample was lyophilized and stored at 4 ℃ for further analysis (Klöppel et al. 2008).

Characterization of Secondary Metabolites

Gas Chromatography – Mass Spectroscopy

The crude extract was analyzed using GCMS (Gas Chromatography Mass Spectrometry) to identify and to confirm the presence of a various compound in them. Helium gas is used as a carrier gas for GCMS, the injection volume was 1 µl with a flow rate of 1.0 µl/min. NIST database spectrum is used for standard. The crude extract spectrum was compared with the NIST database spectrum. The high-intensity chromatogram peak was adjusted with ESI Compass Data Analysis Version 4.0. Moreover, the non-polar column is performed (Bakaraki et al. 2016).

Fourier Transform Infrared Spectroscopy

The lyophilized sample was dissolved in 1 mg/ml of HPLC grade water and subjected to FTIR spectral evaluation. All spectra were recorded within an infrared range from 400 to 4000 cm^{-1} using an FTIR spectrometer (Shimadzu, Japan).

Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a simple yet effective technique for the identification of different groups of secondary metabolites. The crude sample along with the DCM fraction was prepared at a 1 mg/ml concentration. The study was conducted using a silica gel plate for the stationary phase. The crude sample was spotted on silica plate and was transferred to a twin chamber which contains the developing solvent system with water: glacial acetic acid: n-butanol: ethyl acetic acid in the ratio of 1:1:1:1. *P-Anisaldehyde* – sulphuric acid was sprayed and the plate was allowed to dry and then heated to visualize the bands (Al-Massarani et al. 2017).

HPLC Analysis

HPLC was used for the separation and identification of compounds from the crude extract. The lyophilized DCM extract was dissolved in HPLC grade water (1 mg/ml). The sample was analysed with a reverse phase HPLC C18 column and the purified fraction was collected. Initially, the column was washed for 5 min. The crude sample was loaded on the column and the fraction was eluted using methanol and HPLC grade water for 30 min (These et al. 2009).

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

The protein content in the crude sample was determined using SDS-PAGE according to the method of Laemmeli (1970). The crude sample (20 µl) was mixed with Laemmeli loading dye (10 µl) and the mixture was loaded in 10% SDS-PAGE. After 4 hours, the gel was removed from the plates and stained with a staining solution containing 0.1% Coomassie brilliant blue and Methanol: Glacial acetic acid and de-stained with glacial acetic acid: methanol: water (10:30:60). The molecular weight was estimated by comparing it with protein marker and BSA with a molecular weight of 66.5 kDa (He 2011).

Biological Activity

Antibacterial Assay

The antibacterial activity for the crude and DCM fractions of secondary metabolites was determined by Bauer et al. (1966). In this test, human bacterial pathogen cultures namely *Bacillus subtilis* (ATCC 23857), *Pseudomonas, Shigella, Staphylococcus aureus* (ATCC 23235), *Salmonella paratyphi-A* (ATCC 9150) and *Salmonella paratyphi-B* (ATCC BAA-1250/SPB7), *Proteus vulgaris* (ATCC 8427), *Escherichia coli* (ATCC 25922D-5), *Klebsiellapneumoniae* (ATCC BAA-1705D-5) and *Proteus* mirabilis (ATCC 12453) were used in this test. The spread plate technique is employed and followed as per (Bauer 1966). The plates were incubated at 37 °C for 24 hours and observed for clear zones of inhibition formed around the discs, and the diameter of the zones is measured in millimeters (mm). Amoxicillin (10 µl /disc) was used as a positive control.

Antioxidant Assay

Antioxidant activity of the secondary metabolite was evaluated using different assay and the percentage of inhibition was calculated. DPPH radical Scavenging Activity was performed as per Yen and Chan (1995) method (Yen & Chen 1995).The total antioxidant capacity of the crude DCM extract was carried out using the phosphomolybdenum method to determine the total quantity of fat and water-soluble antioxidants presence (Saeed et al. 2012; Singh & Chahal 2018). The ability of secondary metabolite extract to scavenge hydroxyl scavenging was carried out according to the protocol of Halliwell and Gutteridge (Gutteridge & Halliwell 1988).The ability of the crude extract to scavenge hydrogen peroxide is determined as per the method mentioned by Ruch et al. (Ruch et al. 1989).The metal chelating activity of the extract was determined as described by Soler-Rivas (Soler-Rivas et al. 2000). Finally, the percentage of inhibition and the total amount of antioxidants present in the sample were calculated.

Statistical Analysis

Results were expressed as mean SD. The one-way ANOVA followed by Tukey's multiple tests was used to analyze data.

RESULTS AND DISCUSSION

Bacterial Characterization

The bacterial culture isolated from pony fish was gram-negative rod-shaped bacteria when observed microscopically, as shown in Figure 1. In TCBS agar, luminescent green colored colonies were observed macroscopically in the dark, as shown in Figure 2 and green colonies were observed in TCBS agar when provided with a light source as shown in Figure 3. Figures 4 and 5 indicate positive results for catalase and starch in biochemical assays. Gram staining and biochemical analysis revealed that the isolates tested were gram-negative rods, taxonomically identified as *Photobacterium leiognathi*. In the current study, secondary metabolites were extracted from *Photobacterium leiognathi* which was obtained from skin of *S. ruconius*. For *photobacterium*, the tests for catalase and cytochrome oxidase are both positive. Although D-glucose produces acids, it does not produce gas. Nitrate is broken down into nitrite (Nogi et al. 1998).

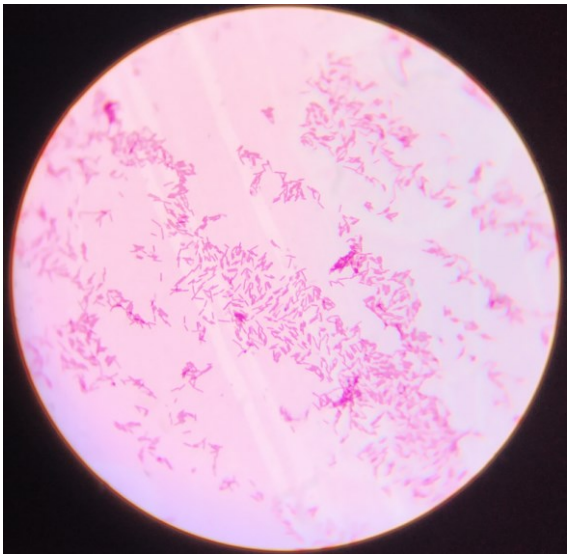

Figure 1. Gram staining – Gram Negative Rods.

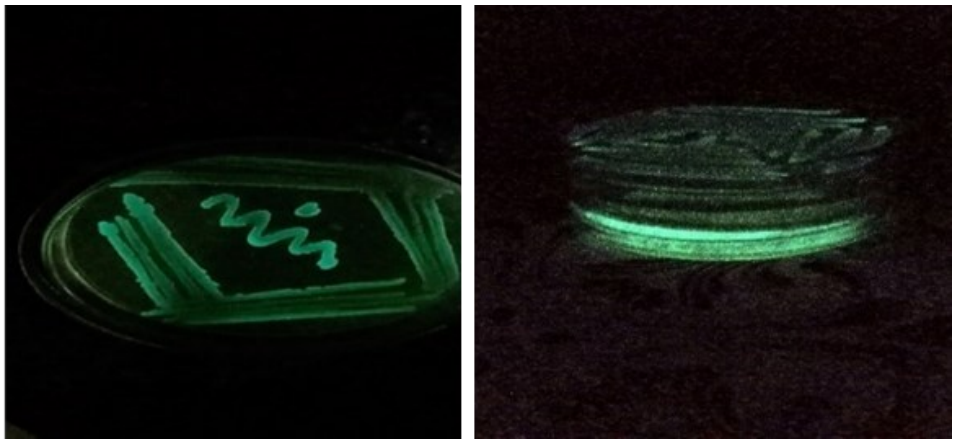

Figure 2. Bioluminescence from *P. leiognathi* in dark.

Gel electrophoresis for PCR product of the sample and a positive control for 16S rRNA gene. The sample and the positive control were amplified by 2% gel electrophoresis as shown in Figure 4.

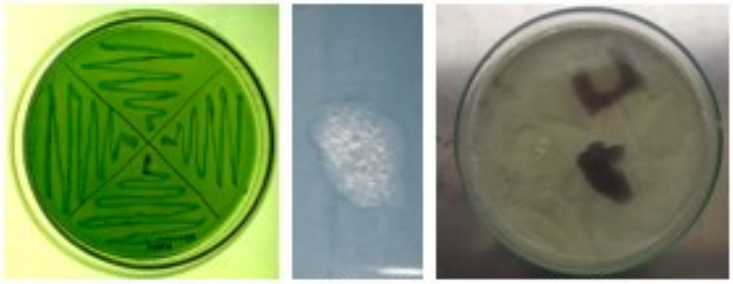

Figure 3. a) Green colour colonies in light (TCBS Agar); b) Catalase test – Positive; c) Starch hydrolysis test - Positive.

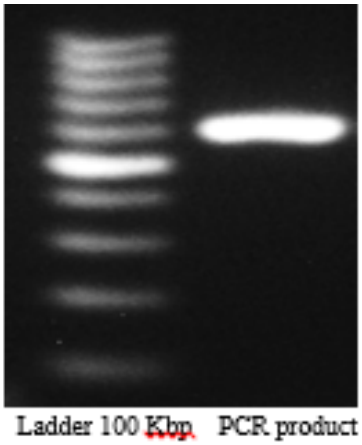

Figure 4. Agarose gel electrophoresis – Left: DNA Ladder 100Kbp, Right: Bacterial DNA.

After sequencing, amplified 16s rRNA of the isolated sample was subjected to BLAST analysis and a phylogenetic tree was constructed with the neighbourhood joining method. The sample of pony fish was found to be similar to that of *P. leiognathi.* The sequence of *P. leiognathi* (ATCC25521) was the closest of the operational taxonomic unit. The species *P. damselae* (ATCC33539) and *P. piscicola* (NCCB100098) have the closest sequence similarity of 99%. The molecular characterization by 16S rRNA sequencing confirmed that the isolated bacterium was a bioluminescent strain of *P. leiognathi* as shown in Figure 5 and was found to be in correlation with similar studies showing the isolation of *P. leiognathi* from pony fish (Molina et al. 2016).

Characterization of Secondary Metabolites

Gas Chromatography – Mass Spectroscopy

GC-MS spectrum of the crude secondary metabolite from the bacterial isolate shows a numerous presence of various components with different retention times as illustrated in Figure 6 and Table 1. Results depicted some of the most common secondary metabolite which were not yet reported from *P. leiognathi* and they are as follow: Cyclopropane, 1-Butyl-2-(2-Methyl Propyl), Oleic acid, Emylcamate, 2-Propenoicn acid, Oxybis (Methyl-2,1-Ethanediyl) ester, 17-(1,5-Dimethyihexyl)-10,13-Dimethyl-1,7,8,9,10,11,12,13,14,15,16,17 -Dodeca,2-Chloropropionic acid, 2,2-Dimethyl Propyl ester, 2,6-Lutidine 3,5-Dichloro-4-Dodecylthio-, 3-Butoxy-1,1,1,5,5,5-Hexamethyl-3-

(Trimethylsicoxy) Trisicoxane. In certain marine bacteria, specific GC-MS compounds such as phenol, dibutyl phthalate, butyl octylester, and indole were observed (Gromek et al. 2016).

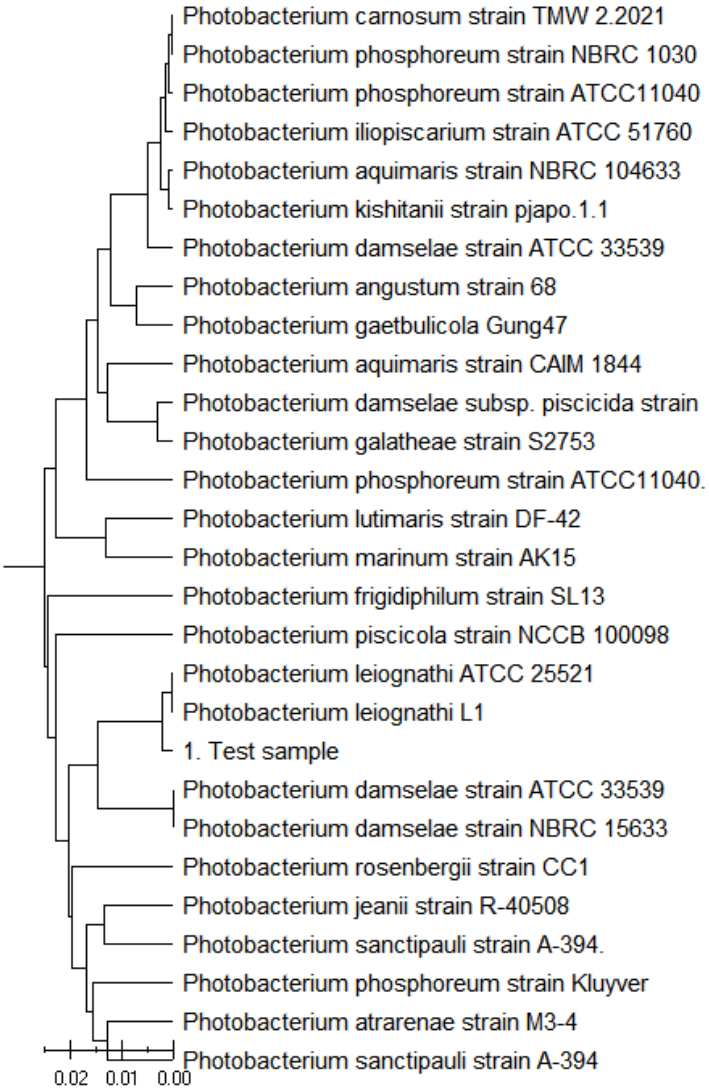

Figure 5. Phylogenetic tree of *P. leiognathi*.

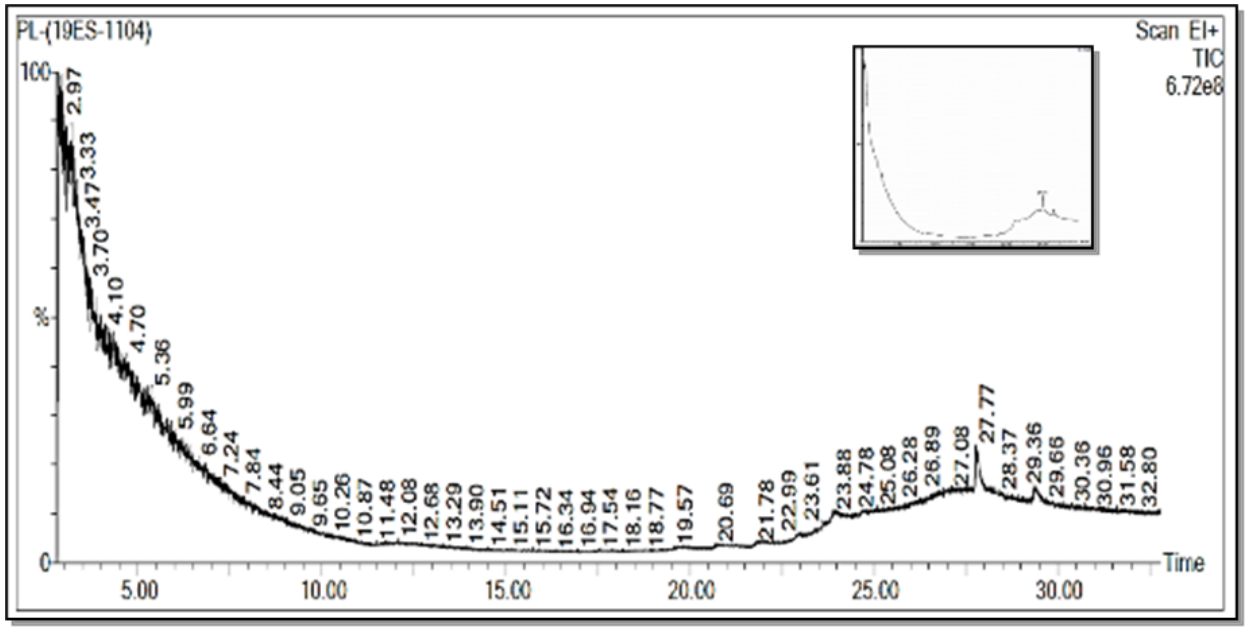

Figure 6. GCMS analysis of secondary metabolites from DCM extract.

Table 1. GCMS analysis of secondary metabolites from DCM extract.

S No.	Retention time	Molecular mass (m/z)	Compound name	Molecular formula
1.	3.083	84	Methylene Chloride	CH_2Cl_2
2.	23.967	154	Cyclopropane,1-Butyl-2-(2-Methyl Propyl)	$C_{11}H_{22}$
3.	24.782	282	Oleic Acid	$C_{18}H_{34}O_2$
4.	25.277	282	Oleic Acid	$C_{18}H_{34}O_2$
5.	26.698	145	Emylcamate	$C_7H_{15}O_2N$
6.	27.138	240	2-Propenoicn Acid, Oxybis(Methyl-2,1-Ethanediyl) Ester	$C_{15}H_{28}O_2$
7.	27.453	382	17-(1,5-Dimethyihexyl)-10,13-Dimethyl-1,7,8,9,10,11,12,13,14,15,16,17-Dodeca	$C_{27}H_{42}O$
8.	27.793	178	2chloropropionic Acid, 2,2-Dimethyl Propyl Ester	$C_8H_{15}0_2Cl$
9.	28.249	375	2,6-Lutidine 3,5-Dichloro-4-Dodecylthio-	$C_{19}H_{31}NC_{12}S$
10.	29.359	368	3-Butoxy-1,1,1,5,5,5-Hexamethyl-3-(Trimethylsicoxy)Trisicoxane	$C_{13}H_{36}O_4S_{14}$

Fourier Transform Infrared Spectroscopy

The lyophilized crude sample was analyzed an FTIR spectrometer and its result is shown the Figure 7. The FTIR spectrum obtained the spectral range of 400 to 4000 cm^{-1} for the active crude sample. Totally, 9 peaks were obtained for crude extract. Apart from 4 peaks, range peaks were also recorded. Infrared spectroscopy is a useful analytical technique for the detection of functional groups of the compound. IR spectrum corresponds to the presence of P-H phosphine at wavelength of 2362.80 cm^{-1}, while the c=c stretching frequency around 1667.78 cm^{-1} conform the alkenes groups and amides group are present in 1548.84 cm^{-1} wave number. A collection of bands in the region of 1408.04 are due to the S=O sulfate group, while the absorption band in the region 1093.64 cm^{-1} are C-F structuring functional group of Ether and alkyl halides functional group present wavelength of 557.43 cm^{-1} structure of C-Br- group. FTIR spectra of the crude extract of the marine bacterium *Pseudomonas aeruginosa* revealed major bands at 3396.01 cm^{-1}, 2928.88 cm^{-1}, 1726.24 cm^{-1}, 1510.88 cm^{-1}, 1726 cm^{-1}, and 1046.43 cm^{-1} (Nair et al. 2021).

Thin Layer Chromatography

The developed TLC plate sprayed with the derivatization agent is shown in

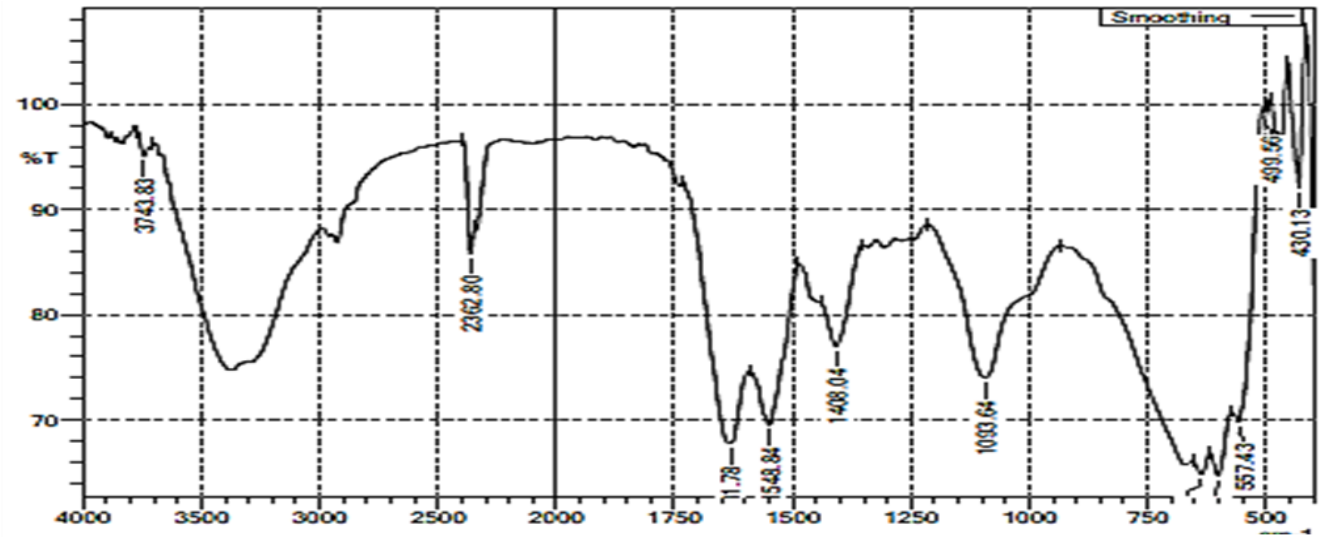

Figure 7. FTIR analysis of DCM extract of bioluminescent bacteria.

figure 8. Crude extract on the right side showed multiple bands with different RF whereas the fraction showed 3 prominent bands in the TLC plate under white light, 254 nm and 366 nm suggesting the presence of individual compounds. Further the colour developed with derivatization agent confirms the presence of functional groups including phenols, sugars, and amino acids in the fraction. TLC bioautography overlay assays were used to detect antimicrobial activity in all fractions using Staphylococcus aureus as the test microorganism, and fraction numbers 13–18 revealed a robust antimicrobial inhibition zone with an Rf value of 0.42 (Zheng et al. 2005).

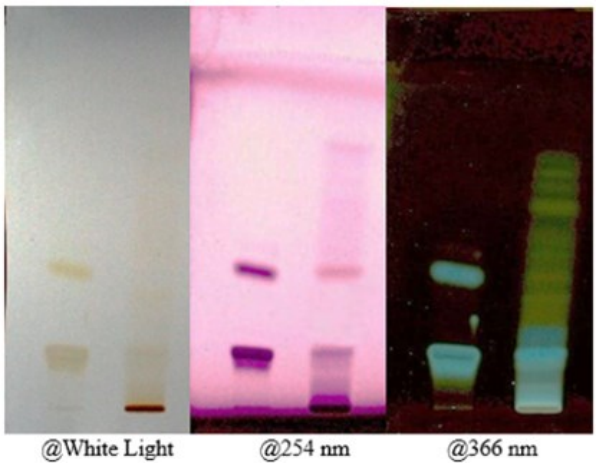

Figure 8. TLC plate analysis of secondary metabolites: A Fraction; B - Crude extract.

High Performance Liquid Chromatography

The DCM extracts were analysed through HPLC. These results showed nine peaks at different retention time and are represented in Figure 9. Among these, the maximum intensity was recorded in the second peak at 7.93 RT. The obtained peak was further purified through preparative HPLC and collected for further analysis. HPLC is generally used for the analytical estimation of various compounds. In the present study, the analysis of DCM extract was performed using reverse phase HPLC column (C-18) with the retention time up to 17.5 min at 258 nm. As a result, nine peaks were observed

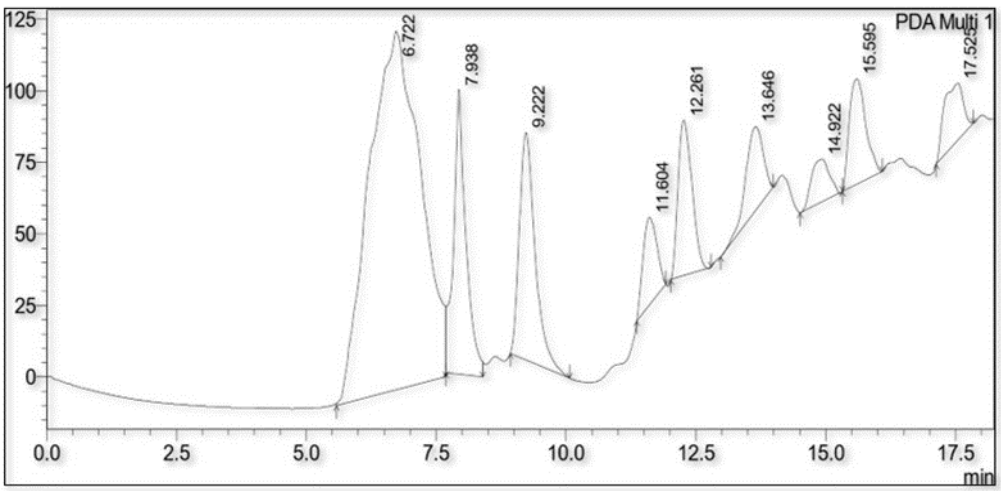

Figure 9. HPLC analysis of secondary metabolites from DCM extract.

with the retention time of 6.72, 7.93, 9.22, 11.60, 12.26, 13.64, 14.92, 15.59 and 17.52. The highest intensity (6.72RT) was observed and further bioactivity evaluations were performed similarly. In the HPLC-PDA, three peaks with maximum absorption wavelengths of 246.5, 281.9, and 337.7 nm were identified in marine bacteria (Zheng et al. 2005).

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

The crude extract of secondary metabolites of *P. leiognathi* was analysed in 10% SDS –PAGE. After electrophoresis, the banding pattern was observed as shown in Figure 10. As a result, nine prominent bands were observed and the same was compared to protein marker with a molecular range between 10 to 203 kDa. Several prominent bands are observed in the molecular range of the crude extract between 10 to 110 kDa. Bacteriocins are proteins (>10 kDa) or short peptides (>10 kDa) that differ greatly from antibiotics (secondary metabolites) in their mode of action and chemical structure (Dobson et al. 2012). Crude toxins in the aqueous extract of *Halichondria panicea* produced nine bands on SDS-PAGE on a 12 percent gel, ranging from 14.3 to 116 kDa, with three well-defined bands at 19.5, 39.0, and 66.2 kDa (Purushottama et al. 2009). To our knowledge, the current study holds the novelty of the first ever report regarding the protein band pattern of DCM extract produced by bioluminescent bacteria, *P. leiognathi*.

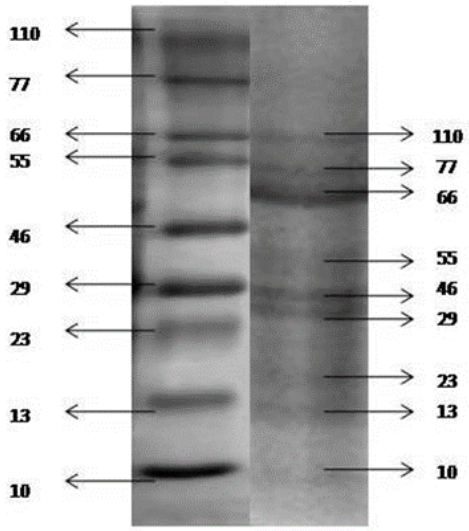

Figure 10. SDS - PAGE analysis of metabolites – 1: Protein Marker, 2: Isolated Proteins

Biological Activity

Antibacterial Assay

The antibacterial activity showed that the crude extract and the fraction exhibited significant antibacterial activity against all ten pathogenic bacteria, as shown in Figure 11 and Table 2. The antibacterial activity was noted for the sample in comparison with the standard antibacterial drug Amoxicillin. The inhibition zone was observed against all pathogenic bacteria in the range of 12 mm to 20 mm for both the crude sample and fraction at a concentration

of 30 µl. However, the maximum inhibition zone of 20±0.32 mm was observed against *Shigella* sp. by crude and 15±0.22 mm by a fraction. Secondary metabolite of the bacterium *P. leiognathi* was extracted using DCM to study its bioactive potential against human pathogens and its antioxidant capacity. The DCM extract showed a broad spectrum of antibacterial activity against the six human bacterial pathogens. The sample at a concentration of 30 µg/ml presented a maximum zone of inhibition against *Shigella* sp. When compared to the standard drug amoxicillin and minimum inhibition towards *P. mirabilis*., Yalla et al. (2018) reported that the ethyl acetate extract (50 mg/ml) of *V. furnissi* showed wide spectrum activity against *S. sonnei* (10.6 mm). The micronutrient-rich medium for the growth of bacteria determines the nature of metabolite produced by them, and thus, the bioactivity varies accordingly (Armstrong et al. 2001; Kelecom 2002). Different antimicrobial compounds produced by *Photobacterium* sp. have been reported. Compounds such as Unarmicins A and C, Holomycin, Ngercheumicin A, B, C, D and E are produced by *Photobacterium* and showed antibacterial and antifungal activities. Antibacterial compounds such as phenol, 2,4-bis (1,1-dimethylethyl) -, Indolizine and 1,2-benzenedicarboxylic acid, butyl octyl ester are produced by *Photobacterium* (Ramesh & Mohanraju 2017). However, these compounds have not been detected in the current study; antibacterial activity was still significant against clinical pathogens.

Table 2. Antibacterial activity of bioluminescent bacteria.

Bacterial pathogen	Inhibition zone in mm		
	Crude (30 µl)	Fraction (30 µl)	Standard (30 µl))
Bacillus subtilis	12±0.32	12±0.36	17±0.18
Salmonella paratyphi-A	12±0.18	12±0.49	14±0.22
Salmonella paratyphi-B	12±0.12	12±0.22	15±0.29
Proteus mirabilis	11±0.49	11±0.31	12±0.11
Proteus vulgaris	12±0.38	13±0.29	13±0.31
Klebsiella pneumoniae	15±0.15	12±0.13	25±0.25
Shigella	20±0.32	15±0.22	30±0.19
Pseudomonas	7±0.37	7±0.24	7±0.21
Staphylococcus aureus	12±0.23	12±0.49	10±0.16
Escherichia coli	17±0.31	10±0.22	20±0.19

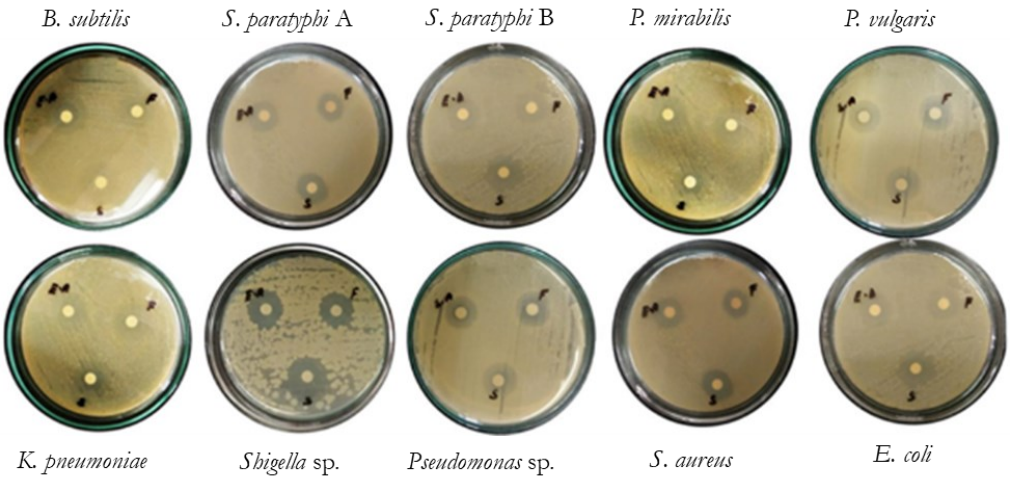

Figure 11. Antimicrobial susceptibility assay for extracts of *P. leiognathi*

Antioxidant Assay

The antioxidant potential of the crude sample was examined using DPPH, HRSA, H2O2, and Metal chelating activity (Figure 12). The results of the antioxidant activity are reported in Table 3. Total antioxidant capacity using the phosphomolybdenum technique is shown in Table 4 Ascorbic acid was used as the standard. The DPPH scavenging activity results showed 98.74±0.18% inhibition for 100 µl of standard and the crude extract showed 86.21±0.29% at 300 µl of concentration. The difference between standard extract and the crude extract is 12.53% which reveals significant DPPH scavenging activity. The hydroxyl radical scavenging activity results showed 98.26±0.21% inhibition for 100 µl of standard and the crude extract showed 83.11±0.31% at 300 µl of concentration. The difference between the standard and the crude extract is 15.55% which reveals significant hydroxyl radical scavenging activity. The results of the hydrogen peroxide scavenging activity showed 89.91±0.19% inhibition for 100 µl of the standard at a concentration of 100 µl and the crude extract showed 81.68±0.34% at 300 µl of concentration. The difference between the standard and the crude extract is 8.23%, which reveals significant hydrogen peroxide scavenging activity. Metal chelating activity results showed 99.12±0.15% inhibition for 100 µl of standard and the crude extract showed 98.45±0.35% at 300 µl of concentration. The difference between the standard and the crude extract is 0.67%, which reveals significant metal chelating activity. The total antioxidant capacity was carried out using the phosphomolybdenum method where a standard ascorbic acid graph was plotted. When 100 µg/ml of the crude extract was evaluated for phosphomolybdenum activity, the optical density when compared to the

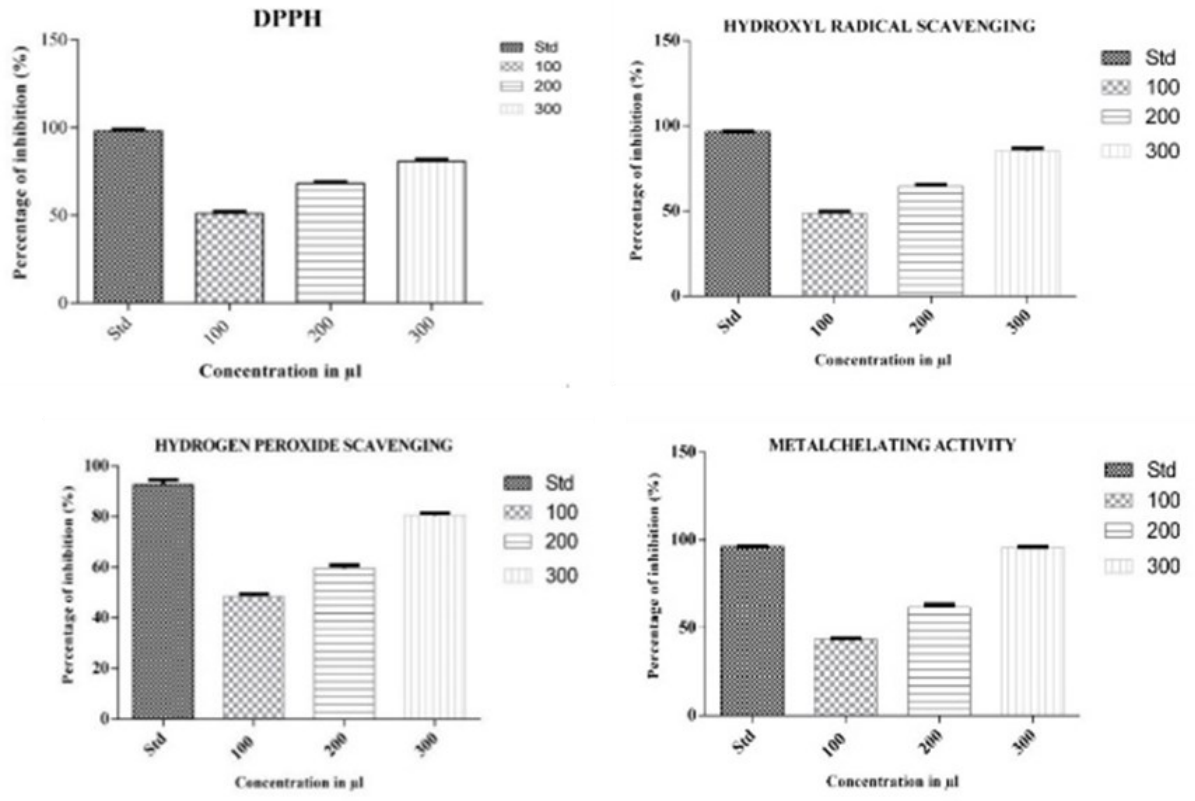

Figure 12. Antioxidant activity of Secondary Metabolites of DCM extracts of bioluminescent bacteria *Photobacterium leiognathi.*

Table 3. Percentage Inhibition of the Crude Extract.

Concentration	Percentage of Inhibition			
	DPPH Scavenging Activity	Hydroxyl Radical Scavenging Activity	Hydrogen Peroxide Scavenging Activity	Metal Chelating Activity
Ascorbic Acid (Standard) – 100µl	98.74±0.18%	98.26±0.21%	89.91±0.19%	99.12±0.15%
Extract – 100µl	40.71±0.34%	39.54±0.48%	49.27±0.28%	47.84±0.22%
Extract – 200µl	68.38±0.41%	62.17±0.32%	59.28±0.22%	53.47±0.18%
Extract – 300µl	86.21±0.29%	83.11±0.31%	81.68±0.34%	98.45±0.35%

standard graph shows that the total antioxidant capacity of the secondary DCM crude extract of the bacterial metabolite to be 50.11 µg/ml. Therefore, out of 100 µg/ml crude extract, 50.11 µg/ml concentration consists of antioxidants revealing that 50% of the extract has antioxidants. The oxidation reaction can produce free radicals which can set off chain reactions that damage cells. Antioxidants stop these chain events by neutralizing or stabilising free radicals. The number of active groups has a favourable relationship with antioxidant activity (OH or NH2) (Chandra et al. 2020). The study has clearly proved that the secondary metabolites identified from the bioluminescent bacteria *P. leiognathi* by GCMS and FTIR have excellent antioxidant properties. The results of the DPPH scavenging activity were supported by (Kumagai et al. 2018). In this activity, crude sample reacts with DPPH and reduces the free radicals of the hydroxyl group (Matthäus 2002). As the activity of DPPH showed a minimum of 40.71±0.34% activity and a maximum of 86.21±0.29% when compared to standard ascorbic acid, the ability to reduce Fe^{3+} ions may be due to the active compound present in the solvent extract (Kekuda et al. 2010). Various disorders related to oxidative stress can be prevented by determining hydrogen peroxide activity (Poongodi et al. 2012). ROS like hydroxyl free radical is produced when hydrogenperoxide reacts with metal ions (Fe2C and/or Cu2C which leads to a toxic effect; in addition, hydrogen peroxide can cross the cell membrane easily (Floyd & Lewis 1983). The reducing capacity of a compound may help to improve cell signaling. The sample serves as a potential antioxidant activity because it has increased reducing power. Although the standard ascorbic acid has a high reducing power compared to that of the extract, the antioxidative property presence is significant for a crude extract without any purification, thus, the extract has the potential to provide electron donors for radical chain reactions.

Table 4. Phosphomolybdenum activity.

Total Antioxidant Capacity - Phosphomolybdenum Method	
Concentration	**Optical Density at 680nm**
Ascorbic Acid – 10 µg/ml	0.067
Ascorbic Acid – 50 µg/ml	0.101
Ascorbic Acid – 100 µg/ml	0.116
Ascorbic Acid – 250 µg/ml	0.195
Ascorbic Acid – 500 µg/ml	0.385
Crude Extract – 100 µg/ml	0.092
Total Antioxidant Capacity – 50.11 µg/ml	

CONCLUSION

Bioluminescence is an area of surging research especially due to its ties with the ocean or the marine environment as many bacteria and other organisms either in their free-state or symbiotic relationship express bioluminescence which can be used for the human welfare development. To apply bioluminescence for industrial usage, isolation of bioluminescence bacteria must be carried out from different sources based on the stable light intensities. The current study is the first successful attempt to isolate *P. leiognathi* from *S. ruconius* and their morphological, biochemical, and molecular identification was documented. Then, crude secondary metabolites were extracted using dichloromethane, and their compositional and functional group analysis were done using GC-MS and FTIR. Cyclopropane, 1-Butyl-2-(2-Methyl Propyl), Oleic acid, Emylcamate, 2-Propenoicn acid, Oxybis(Methyl-2,1-Ethanediyl) ester etc., were the functional group identified by GCMS. The FTIR spectrum obtained the spectral range of 400 to 4000 cm^{-1} for the active crude sample. Totally, 9 peaks were obtained for crude extract. The extracts were also characterized using chromatographic techniques, including TLC and HPLC, finally, protein analysis was reported using SDS-PAGE. TLC confirms the presence of functional groups including phenols, sugars, and amino acids in the fraction. In HPLC, nine peaks were observed with a retention time of 6.72, 7.93, 9.22, 11.60, 12.26, 13.64, 14.92, 15.59 and 17.52. The metabolites were then profiled for their antibacterial activity against ten different clinical bacterial pathogens and showed excellent activity. Another important bioactivity that was extensively carried out was the antioxidant activity and total antioxidant capacity of the secondary metabolites. One of the most important findings is that the secondary metabolites showed excellent antioxidant activity when studied using different assays and the analysis of the total antioxidant capacity revealed that 50% of the extract contains rich antioxidants. These results will be instrumental in developing novel products with biosensor and bio imaging applications using *P. leiognathi*.

AUTHORS CONTRIBUTION

S.T. performed the experiments, wrote, analyzed and interpreted the data. U.S. and S.R. analyzed the data, K.S. discussed the article. A.M. designed, supervised physicochemical experiments, and discussed the article.

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CONFLICT OF INTEREST

The authors report that there is no conflict of interest.

REFERENCES

Al-Massarani, S.M. et al., 2017. Isolation, biological evaluation, and validated HPTLC quantification of the marker constituent of the edible Saudi plant Sisymbrium irio L. *Saudi Pharmaceutical Journal*, 25(5), pp.750–759. doi: 10.1016/j.jsps.2016.10.012

Armstrong, E. et al., 2001. The symbiotic role of marine microbes on living surfaces. *Hydrobiologia*, 461(1), pp.37–40. doi: 10.1023/A:1012756913566

Bakaraki, N. et al., 2016. Development of a sensitive liquid--liquid extraction method for the determination of N-butyryl-l-homoserine lactone produced in a submerged membrane bioreactor by gas chromatography mass spectrometry and deuterated anthracene as the internal standard. *Analytical Methods*, 8(12), pp.2660–2665. doi: 10.1039/C6AY00317F

Bauer, A.W., 1966. Antibiotic susceptibility testing using a standardized single disc method. *Am J clin pathol*, 45, pp.149–158. doi: 10.1128/am.13.2.279-280.1965.

Chandra, P., Sharma, R.K. & Arora, D.S., 2020. Antioxidant compounds from microbial sources: A review. *Food Research International*, 129, p.108849. doi: 10.1016/j.foodres.2019.108849

Dobson, A. et al., 2012. Bacteriocin production: a probiotic trait? *Applied and environmental microbiology*, 78(1), pp.1–6. doi: 10.1128/AEM.05576-11

Engebrecht, J., Nealson, K. & Silverman, M., 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from Vibrio fischeri. *Cell*, 32(3), pp.773–781. doi: 10.1016/0092-8674(83)90063-6

Farrelly, V., Rainey, F.A. & Stackebrandt, E., 1995. Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Applied and environmental microbiology*, 61(7), pp.2798–2801. doi: 10.1128/aem.61.7.2798-2801.1995

Firudoz, B. et al., 2020. Molecular variation and phylogenetic status of ponyfish (Perciformes: Leiognathidae) in Karaikal, South India. *Notulae Scientia Biologicae*, 12(2), pp.251–257. doi: 10.15835/nsb12210661

Floyd, R.A. & Lewis, C.A., 1983. Hydroxyl free radical formation from hydrogen peroxide by ferrous iron-nucleotide complexes. *Biochemistry*, 22 (11), pp.2645–2649. doi: 10.1021/bi00280a008

Gromek, S.M. et al., 2016. Leisingera sp. JC1, a bacterial isolate from Hawaiian bobtail squid eggs, produces indigoidine and differentially inhibits vibrios. *Frontiers in microbiology*, 7, p.1342. doi: 10.3389/fmicb.2016.0134

Gutteridge, J.M. & Halliwell, B., 1988. The deoxyribose assay: an assay both for'free'hydroxyl radical and for site-specific hydroxyl radical production. *Biochemical Journal*, 253(3), p.932. doi: 10.1042/bj2530932

Haddock, S.H.D., Moline, M.A. & Case, J.F., 2010. Bioluminescence in the sea. *Annual review of marine science*, 2, pp.443–493. doi: https://doi.org/10.1146/annurev-marine-120308-081028

He, F., 2011. Laemmli-sds-page. *Bio-protocol*, e80. doi: 10.21769/BioProtoc.80

Kannahi, M. & Sivasankari, S., 2014. Isolation and identification of bioluminescent bacteria from marine water at Nagapattinam sea shore area. *Int. J. Pharm. Sci. Rev. Res*, 26(2), pp.346–351.

Kekuda, P.T.R. et al., 2010. Studies on antioxidant and anthelmintic activity of two Streptomyces species isolated from Western Ghat soils of Agumbe, Karnataka. *Journal of Pharmacy Research*, 3(1), pp.26–29.

Kelecom, A., 2002. Secondary metabolites from marine microorganisms. *Anais da Academia Brasileira de Ciências*, 74(1), pp.151–170. doi: 10.1590/S0001-37652002000100012.

Kim, S.U. et al., 2018. Adenosine triphosphate bioluminescence-based bacteria detection using targeted photothermal lysis by gold nanorods. *Analytical chemistry*, 90(17), pp.10171–10178. doi: 10.1021/acs.analchem.8b00254. Epub 2018 Aug 20.

Klöppel, A. et al., 2008. HPTLC coupled with bioluminescence and mass spectrometry for bioactivity-based analysis of secondary metabolites in marine sponges. *JPC-Journal of Planar Chromatography-Modern TLC*, 21(6), pp.431–436. doi: 10.1556/jpc.21.2008.6.7

Kumagai, M. et al., 2018. Antioxidants from the brown alga Dictyopteris undulata. *Molecules*, 23(5), p.1214. doi: 10.3390/molecules23051214

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, pp.680-685. https://doi.org/10.1038/227680a0

Matthäus, B., 2002. Antioxidant activity of extracts obtained from residues of different oilseeds. *Journal of Agricultural and Food Chemistry*, 50(12), pp.3444–3452. doi: 10.1021/jf011440s

Menz, J., Schneider, M. & Kümmerer, K., 2013. Toxicity testing with luminescent bacteria--characterization of an automated method for the combined assessment of acute and chronic effects. *Chemosphere*, 93(6), pp.990–996. doi: 10.1016/j.chemosphere.2013.05.067

Molina, A.J., Abisado, R.G. & Calugay, R.J., 2016. Bioluminescent Vibrio spp. with antibacterial activity against the nosocomial pathogens Staphylococcus aureus and Klebsiella pneumoniae. *AACL Bioflux*, 9(2), pp.185-194.

Morin-Crini, N. et al., 2019. Applications of chitosan in food, pharmaceuticals, medicine, cosmetics, agriculture, textiles, pulp and paper, biotechnology, and environmental chemistry. *Environmental Chemistry Letters*, 17 (4), pp.1667–1692. doi: 10.1007/s10311-019-00904-x

Nair, A. V, Vijayan, K.K. & others, 2021. Antibacterial assay guided isolation of a novel hydroxy-substituted pentacyclo ketonic compound from Pseudomonas aeruginosa MBTDCMFRI Ps04. *Brazilian Journal of Microbiology*, 52(1), pp.335–347. doi: 10.1007/s11033-021-06146-x

Nogi, Y., Masui, N. & Kato, C., 1998. Photobacterium profundum sp. nov., a new, moderately barophilic bacterial species isolated from a deep-sea sediment. *Extremophiles*, 2(1), pp.1–8. doi: 10.1007/s007920050036

Nunes-Halldorson, V. da S. & Duran, N.L., 2003. Bioluminescent bacteria: lux genes as environmental biosensors. *Brazilian journal of Microbiology*, 34, pp.91–96. doi: 10.1590/S1517-83822003000200001

Poongodi, S. et al., 2012. Marine actinobacteria of the coral reef environment of the gulf of mannar biosphere reserve, India: a search for antioxidant property. *Int. J. Pharm. Pharm. Sci*, 4, pp.316–321.

Purushottama, G.B. et al., 2009. Bioactivities of extracts from the marine sponge Halichondria panicea. *Journal of Venomous Animals and Toxins including Tropical Diseases*, 15(3), pp.444–459. doi: 10.1590/S1678-91992009000300007

Ramesh, C.H. & Mohanraju, R., 2017. Antibacterial activity of marine bioluminescent bacteria. Indian Journal of Geo Marine Sciences. Vol. 46 (10), pp. 2063-2074

Reysenbach, A.-L. & Pace, N.R., 1995. Reliable amplification of hyperthermophilic archaeal 16S rRNA genes by the polymerase chain reaction. *Archaea: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY*, pp.101–107. doi: 10.1023/A:1015122926687

Ruch, R.J., Cheng, S. & Klaunig, J.E., 1989. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis*, 10(6), pp.1003–1008. doi: 10.1093/carcin/10.6.1003

Saeed, N., Khan, M.R. & Shabbir, M., 2012. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts Torilis leptophylla L. *BMC Complementary and alternative medicine*, 12(1), pp.1–12. doi: 10.1186/1472-6882-12-221

Sarkar, M.K.D., Ahmmed, T. & others, 2019. Antibiotic resistance analysis of Vibrio spp. isolated from different types of water sources of Bangladesh and their characterization. *Eur J Med Health Sci*, 1, pp.19–29. doi: 10.34104/ejmhs.01929

Sharifian, S. et al., 2018. The emerging use of bioluminescence in medical research. *Biomedicine \& Pharmacotherapy*, 101, pp.74–86. doi: 10.1016/j.biopha.2018.02.065

Singh, R. & Chahal, K.K., 2018. Phytochemical analysis and in vitro antioxidant capacity of different solvent extracts of Saussurea lappa L. roots. *J Pharma Phytochem*, 7(3), pp.427–432. doi: 10.1016/j.sjbs.2022.01.040

Soler-Rivas, C., Esp\'\in, J.C. & Wichers, H.J., 2000. Oleuropein and related compounds. *Journal of the Science of Food and Agriculture*, 80(7), pp.1013–1023. doi: 10.1002/(SICI)1097-0010(20000515)80:7<1013::AID-JSFA571>3.0.CO;2-C

These, A., Scholz, J. & Preiss-Weigert, A., 2009. Sensitive method for the determination of lipophilic marine biotoxins in extracts of mussels and processed shellfish by high-performance liquid chromatography--tandem mass spectrometry based on enrichment by solid-phase extraction. *Journal of Chromatography A*, 1216(21), pp.4529–4538. doi: 10.1016/j.chroma.2009.03.062. Epub 2009 Mar 27.

Yalla, S.K., Cherian, T. & Mohanraju, R., 2018. Antimicrobial potential of secondary metabolites extracted from Vibrio furnissii, a luminescent bacterium associated with squid, Uroteuthis duvauceli. *Int. J. of Pharm. and Biol. Sci*, 8(1), pp.530–534.

Yen, G.-C. & Chen, H.-Y., 1995. Antioxidant activity of various tea extracts in relation to their antimutagenicity. *Journal of agricultural and food chemistry*, 43(1), pp.27–32. doi: 10.1021/jf00049a007

Zheng, L. et al., 2005. Antimicrobial screening and active compound isolation from marine bacterium NJ6-3-1 associated with the sponge Hymeniacidon perleve. *World Journal of Microbiology and Biotechnology*, 21(2), pp.201–206. doi: 10.1007/s11274-004-3318-6