Journal of Tropical Biodiversity and Biotechnology
Volume 09, Issue 03 (2024): jtbb90693
DOI: 10.22146/jtbb.90693

Research Article

Spongia officinalis -Associated *Pseudomonas fluorescens* as a Reservoir of Bioactive Compounds: A Novel Source of Natural Anticancer Compounds

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Keywords:
Anticancer activity
Antioxidant
HeLa cell line
Marine sponge
Pseudomonas fluorescens
Spongia officinalis
Submitted:
14 November 2023
Accepted:
05 April 2024
Published:
07 August 2024
Editor:
Ardaning Nuriliani

ABSTRACT

Marine sponges are important sources of chemical variety and repository of biodiversity. In this study, the microbial communities found in the *Spongia officinalis* that was taken from the Kanyakumari coast in India were explored. We identified, characterised, and evaluated the bioactive potential of the sponge-associated bacteria. A total of 12 bacterial isolates were obtained, primarily consisting of gram-positive rods (7 isolates) and some gram-negative rods (2 isolates), and cocci (1 isolate). Among these KKS6 showed tremendous radical scavenging activity (85.16 ±1%) with a minimum inhibitory concentration as 167.26±0.1 µg/mL at the highest concentration when compared to other extracts. With an IC50 value of 55.32 g/mL, this isolate also displayed impressive anticancer activity against HeLa cells. The screened isolate was identified as *Pseudomonas fluorescens* strain using 16S rRNA sequencing. This discovery emphasises the importance of bacteria associated with *Spongia officinalis* as a source of bioactive compounds with medicinal potential. This study highlights the novel findings of diverse microbial communities found in *Spongia officinalis* and their potential for use in biotechnology and medication development. *Pseudomonas fluorescens* was found to be a prolific generator of bioactive byproducts, including strong antioxidants and anticancer agents, which could be a potent drug molecule in future anticancer research.

INTRODUCTION

The marine environment is the largest source of unexplored chemical richness, which has piqued the interest of health scientific communities (Karthikeyan et al. 2022). The tremendous marine biodiversity, which consists of some 230,000 known species and the bioactives constitutes a vast reservoir of anticancer drugs, with a market value estimated to be within USD 5.69 trillion (Wang et al. 2020). Notably, bacteria located within these sponges have been discovered to have a critical role in the manufacture of bioactive chemicals with various pharmacological characteristics (Srinivasan et al. 2021). Marine sponges have a great diversity of bacterial populations which are assumed to be deeply engaged in their

host sponges' metabolic activities (Taylor et al. 2007; Brinkmann et al. 2017).

The search for novel chemotherapeutic drugs has prompted scientists to investigate the isolation, identification, and characterisation of these metabolites, with a particular focus on their anticancer action (Mohan et al. 2022). The marine environment offers a unique and mostly untapped resource for such compounds, the complicated symbiotic connections between marine sponges and their accompanying bacteria hold the possibility of revealing a treasure trove of pharmacologically relevant chemicals. This has sparked a surge of interest in studying the isolation and identification of these bacterial strains, as well as the processes through which they synthesise physiologically active chemicals (Fenical 1993).

The Demospongiae *Spongia officinalis*, is a rich source of secondary metabolites, particularly sterols, terpenoids and phenol (Migliuolo et al. 1990; Manzo et al. 2011). It has also been demonstrated that *S. officinalis* supports a substantial and varied bacterial community. Moreover metabolites retrieved from *Spongia officinalis* have proven to show a wide variety of medical potential (Stabili et al. 2008; Prastiyanto et al. 2022).

The current work conducts a thorough investigation into the isolation, identification, and characterisation of bacterial symbiont within marine sponge *Spongia officinalis*, with a focus on the bioactive metabolites of symbiont bacteria and their potential as an anticancer lead candidate.

MATERIALS AND METHODS

Collection of sponges from coastal area

In the southernmost point of peninsular India, Kanyakumari (N 8° 5' 5.694", E 77° 32' 30.4656), marine sponge was collected by SCUBA diving at a depth of ten feet by random sampling. With latex gloves on, the samples were cut using a dive knife. The pieces were then placed into different plastic sample collection bags, and then transported within two hours to a laboratory for further processing. The salinity, temperature, pH, and TDS (Total Dissolved Solids) were determined to be 31.24%, 29.8°C, 7.89 and 312 mg/L respectively (Jin et al. 2014).

Processing of sponges

Sponge tissues were cleaned with sterile and were cut with a sterile scalpel blade from the inner mesohyl. The sterile 1-cm^3 sponge sample was pulverised vigorously for two to three minutes in a sterile mortar with 9 mL of sterile sea water (Cheng 2017).

Isolation of bacteria from sponge sample

The homogenized sponge sample was serially diluted to a concentration of 10^{-6} in sterile seawater, and 100 µl aliquots were plated onto Zobell marine agar medium. The plates were incubated at 37°C for 48 hrs. Colonies were selected from isolation plates and re-streaked at least twice to achieve pure cultures from single colonies from plates that were visually considered to be from single cultures. Cultures were incubated at 37°C in zobell marine broth made with artificial seawater (Webster & Hill 2001).

Morphological identification of sponge associated bacteria

A method suggested by (Photolo et al. 2020) was applied to determine the shape and gram stain reaction. To determine morphological traits including form and Gram stain reaction, pure colonies were subjected to Gram staining. Using a compound light microscope with a 100x magnification, Gram stain slides were examined.

Extraction of crude secondary metabolite from bacteria

The pure colonies of bacterial isolates were inoculated into 10 mL of zobell marine broth and incubated at 37 °C with shaking for three days to create seed cultures. In a 250 mL conical flask, 50 mL of zobell marine broth was inoculated with about 1 mL of these seed cultures. These were incubated for three days at 37°C in a shaking incubator (Kennedy et al. 2009). Bacterial cells were removed during the preparation of extracts by centrifugation at 3000 x g for 15 min. By employing liquid-liquid chromatography with ethyl acetate as a solvent, the cleared culture broth was used to isolate metabolites. The extract was evaporated in a rotary evaporator set at 40 °C and 90 rpm in order to extract the crude metabolites. The leftover material was recondensed. After that, the concentrated crude extract was kept at 4 °C for further research (Arasu et al. 2014).

Preliminary screening of chemical compounds

Standard qualitative chemical analysis was conducted on metabolite extract of isolates to detect the presence of chemical compounds such as phenol, alkaloid, flavonoid, terpenoid, steroid, saponins, tannins and glycoside (Harborne 1973).

Bioactivity of sponge associated microbial metabolite

Antioxidant activity of extracted metabolite

A sterile 96-well plate was used to test the metabolite capacity to scavenge DPPH free radicals. The extract was blended (1:1 v/v) with DPPH (0.02 mg/mL) at various doses and incubated at 25 °C in the dark for 30 minutes. The absorbance was measured at 517 nm using an ELISA plate reader (The Infinite F50 Plus-with Magellan data analysis software). Ascorbic acid (AA) was utilised as thestandard. The percent inhibition of DPPH radical was measured by using the formula: [A0 − A1 / A0] × 100 where, A1 and A0 equal the absorbance of the control and the test, respectively and median inhibitory concentration (IC50) was calculated (Tsilo et al. 2020).

Anticancer activity- MTT assay

HeLa cells were seeded in Roswell Park Memorial Institute (RPMI) 1640 medium, and the initial number of cells was counted using a microscope. After being trypsinised, the cells were centrifuged. A hemocytometre was used to count the cells after the supernatant was removed and the pellet was resuspended in 1 mL of complete media. A 96-well plate containing 2×10^4 cells was then seeded with 100 µl of the medium and cultured overnight to promote cell adhesion. Following that, 100 µL of test material extracts were added to each well at varied concentrations (512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 µg/mL). The cells were then kept at 37°C for another 24 hours. The cells were examined under a microscope after 24 hours. Each well was treated with 5-mg/mL 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT). To dissolve the formazan crystals, a stop solution of dimethyl sulfoxide (DMSO) was then given to each well. An ELISA plate reader (The Infinite F50 Plus-with Magellan data analysis software) was used to measure the cell viability at 570 nm (Elmanama et al. 2020).

Biochemical and molecular identification of bacteria

The bacterial isolates were identified using methyl red, indole, vogues proskauer, urease,citrate utilisation, TSI and oxidase test (Chelossi et al. 2004). The DNA isolation was carried out by phenol-chloroform methodIn brief, a homogenization buffer containing proteinase K and

SDS was used for cell lysis. The DNA-rich aqueous phase was then obtained through phase separation using phenol, chloroform, and isoamyl alcohol, and was further precipitated with isopropanol. The extracted DNA was redissolved in a solution containing Tris and EDTA and kept at -20°C until use (Wright et al. 2017). 16S rRNA sequencing was used to identify the isolated DNA from the bacterial isolate. The template for PCR amplification was genomic DNA. For 16S rRNA gene amplification primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') were used. The following conditions were used for the PCR process: initial denaturation at 94°C for 5 min, then 40 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 3 min, with a final extension at 72°C for 10 min. dNTPs (0.2 mM each), 1 reaction buffer (20 mM Tris pH 8.4, 50 mM KCl), MgCl2 (1.7 mM), primers (0.1 M each), and Taq DNA polymerase (1.25 units) were included in the PCR reaction mixture (50 µl). Amplification was done using a Thermocycler system (HiMedia Laboratories Private Limited). Agarose gel electrophoresis was used to evaluate the PCR products (Kennedy et al. 2009).The PCR fragments were sequenced by Genetic analyser (Sanger DNA Sequencer). The 16S rRNA gene sequence was evaluated using BLAST NCBI GenBank database to identify similar bacterial sequences. The phylogenetic tree was created using MEGA 7 software, according to Neighbor Joining method.

RESULTS AND DISCUSSION

Sponge identification isolation of bacteria

Marine sponge, *Spongia officinalis* (class Demospongiae) (Figure 1) was identified at the Department of Zoology, University of Madras. Demosponges have reportedly been found to contain a significant amount of pharmaceutically relevant bioactive chemicals (Krishnan & Keerthi 2016). The total colony-forming units of sponge's sample were analysed four days after initial inoculation. Totally 12 different bacterial colonies were isolated from processed marine sponge sample on the basis of unique colonial characteristics on Zobell marine agar plate. The 12 isolates were labelled as KKS01, KKS02, KKS03, KKS04, KKS05, KKS06, KKS07, KKS08, KKS09, KKS10, KKS11, KKS12 respectively.

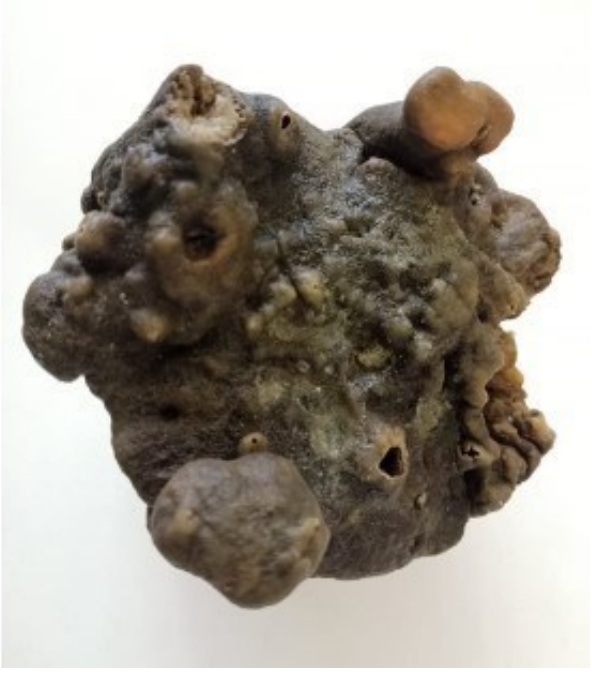

Figure 1. *Spongia Officinalis* (class Demospongiae).

Morphological identification of metabolites

Morphological helps in partial identification of microorganism. Gram staining and colony morphology (colour, form, margin, elevation) results of all the 12 isolates were represented in Table 1 and Figure 2. Colony morphological characteristics of the isolates showed that all the isolate have shown varied morphologies. Gram staining results revealed that gram positive strain dominated the isolates extracted from *Spongia officinalis*.

Table 1. Colony Morphology and Gram Staining of Selected Isolates.

S.NO	Isolate	Colony Morphology	Gram Reaction
1	KKS01	Orange, irregular, convex and entire	Gram positive rod
2	KKS02	White, punctiform, convex, smooth	Gram positive rod
3	KKS03	Yellow, circular, flat, wavy	Gram positive rod
4	KKS04	Creamy white, round, convex, entire	Gram negative rod
5	KKS05	Yellowish white, irregular, raised, curled	Gram positive rod
6	KKS06	Yellowish green, round, raised and smooth	Gram negative rod
7	KKS07	Pale white, flat, convex, entire	Gram positive rod
8	KKS08	White, circular, raise, entire	Gram positive rod
9	KKS09	Off white, irregular, convex, wavy	Gram negative rod
10	KKS10	Orange, circular, flat, entire	Gram negative rod
11	KKS11	Yellow, punctiform, convex, entire	Gram negative cocci
12	KKS12	Glossy white, circular, raised, wavy	Gram positive rod

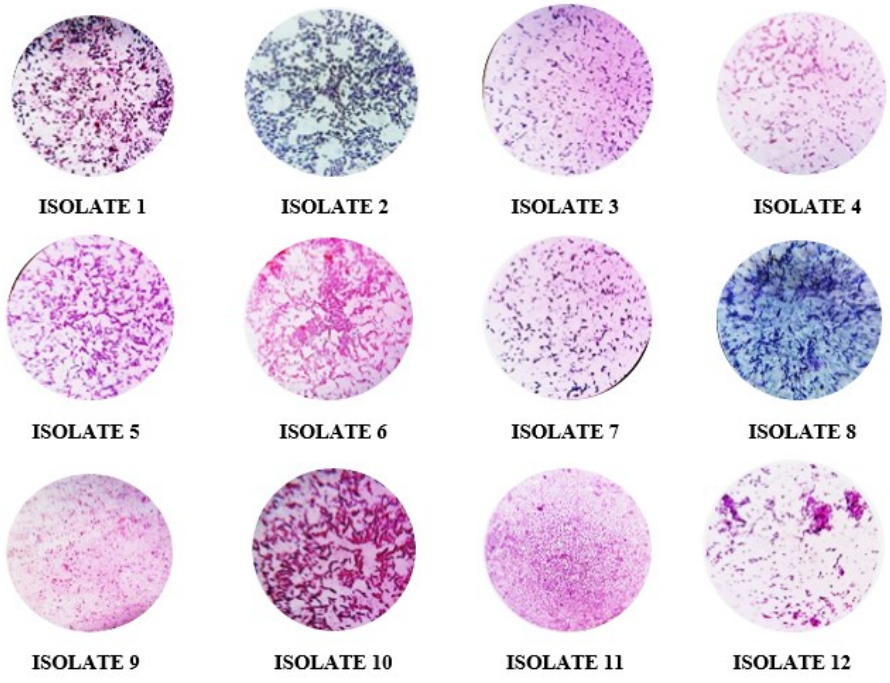

Figure 2. Gram Staining of Bacterial Isolates.

Extraction of secondary metabolites from bacteria using liquid-liquid extraction method

Potential bacterial strains were produced using the flask method using Zobell marine broth for 3 days. To determine a good yield on alternate days, culture was analysed. Broth contains a higher yield of secondary metabolites based on the quantity of yield at the fifth day of culture. The crude ethylacetate of 12 strains extract was subjected to preliminary bioactive compound screening. The production capacity of secondary metabolites often depended on a stationary phase of bacterial development, during which there was a steady overall rate of bacterial growth and death. Active secondary metabolite with antioxidant capacity was screened further by DPPH assay.

Preliminary screening of bioactive compounds

Qualitative chemical analysis

The qualitative chemical analysis of ethylacetate extract of 12 isolates are represented in table 2. Table 2 explains the 8 different bioactive metabolites present in ethylacetate extracts of isolates by qualitative analysis. Different bioactive compounds were present in different isolates. Commonly, phenol and flavonoid were unanimously present in almost all isolates except isolates KKS03 and KKS12. Triterpenoids from several sea sponges have been reported to have cytotoxic action (Li et al. 2013). Marine sponges contain alkaloids, steroids, terpenoids, phenols, and saponins that have been linked to a variety of bioactivities, including antibacterial and antioxidant properties (Riguera 1997; Bhakuni & Rawat 2006; Tangman et al. 2015). From the result, it was evident that all these chemical compounds either individually or in combination contribute to various health benefits.

Screening of anticancer potential metabolites

DPPH assay

Reactive Oxygen Species (ROS) overproduction is a condition that leads to oxidative stress. Oxidative stress, which has been connected to several

Table 2. Phytochemical analysis of ethylacetate extract of selected isolates.

Isolate	Phenol	Flavanoid	Alkaloid	Tannins	Saponnin	Glycoside	Steroid	Terpenoid
KKS01	++	++	-	-	+	-	-	-
KKS02	+++	++	-	-	++	-	-	
KKS03	-	-	++	-	-	-	-	+
KKS04	++	++	+	-	-	-	-	-
KKS05	+++	++	-	-	-	-	-	-
KKS06	+++	+++	-	+	-	-	++	+++
KKS07	-	-	-	++	++	-	++	-
KKS08	+++	+++	-	-	-	-	-	-
KKS09	+	+	-	-	-	++	++	-
KKS10	+	+++	-	+	-	-	-	-
KKS11	+	+	-	-	-	-	-	-
KKS12	-	-	+++	-	++	-	++	-

Note: '-' Indicates absence, '+' Indicates slight presence, '++' Indicates moderate presence, '+++' Indicates High Presence.

health problems, including cancer, is defined as a disparity among the body's enzymatic antioxidants and the rate at which free radicals are produced. According to a number of recent studies, bacterial metabolites function as antioxidants by producing certain compounds that can scavenge oxygen radicals. The ethyl acetate extracts from 12 bacterial isolates have been tested for antioxidant activity by DPPH assay. Isolate KKS02, KKS04, KKS05, KKS07, KKS08, KKS09, KKS10, KKS11 and KKS12 did not show good antioxidant activity. Figure 3 and 4 represents the percentage inhibition of ascorbic acid and ethyl acetate extract of 12 isolates against DPPH to analyse the antioxidant activity.

Figure 3 shows the percentage inhibition of standard ascorbic acid at 1000µg/mL was found to be 90.32±0.2, while the IC_{50} value was observed to be 99.05±0.3 According to Figure 4, the percentage inhibition was observed to be 80.73±0.1, 52.86±1, 73.01±0.8, 52.10±0.9, 53.85±0.9, 85.16±1, 53.73±0.9, 42.70±0.8, 41.35±0.3, 35.98±0.3, 45.44±0.5, 39.13±0.2 at 1000µg/mL for KKS01, KKS02, KKS03, KKS04, KKS05, KKS06, KKS07, KKS08, KKS09, KKS10, KKS11 and KKS12 respectively. In addition, IC_{50} values of KKS01, KKS02, KKS03, KKS04, KKS05, KKS06, KKS07, KKS08, KKS09, KKS10, KKS11 and KKS12 were found to be 237.24±0.6, 887.08±0.7, 322.92±0.8, 897.83±0.8, 832.26±0.8, 167.26±1, 740.90±0.6, 1144.67±0.7, 1202.92±0.5, 1372.31±0.4, 1056.52±0.6, 1341.30±0.5 respectively. Among these KKS6 showed tremendous radical scavenging activity (85.16 ±1%) with minimum inhibitory concentration of 167.26±0.1 µg/mL at highest concentration when compared to other extracts. In this study, the metabolite extracted from KKS6 isolates showed strong antioxidant activity. According to earlier research, *Bacillus licheniformis* established antioxidant molecules from shrimp waste that were highly effective antioxidants (Kumar et al. 2013). Similarly, Extracts of *Actinobacteria* isolated from soil or marine sediments have been proven in studies to exhibit potent antibacterial and antioxidant properties (Rao & Rao 2013; Shivale et al. 2018). The antioxidant capacity of the crude metabolites would have been enhanced by the presence of bioactive chemicals. As the bioactive metabolites demonstrated a potential response in scavenging the free radicals, it may be used as a useful medication to treat pathological illnesses caused by free radicals, such as cancer (Arunachalam & Appadorai 2013).

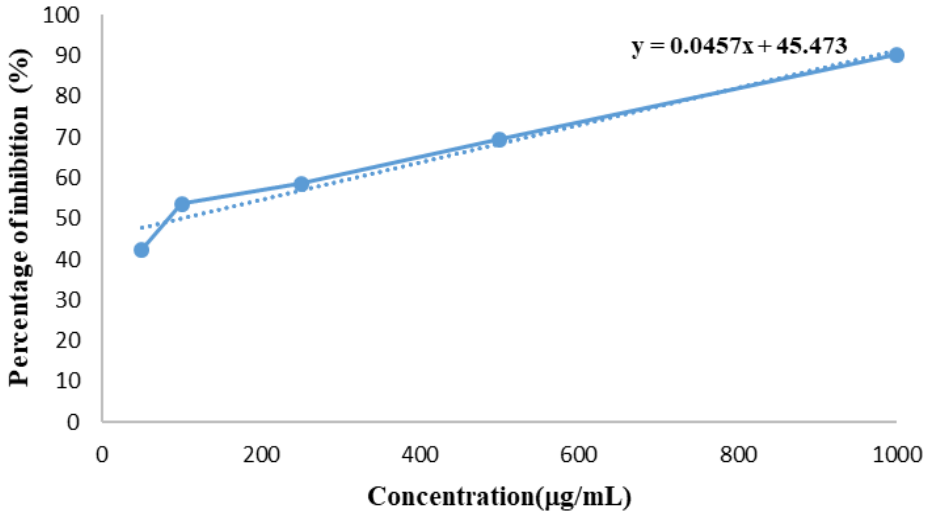

Figure 3. Percentage of inhibition (DPPH ASSAY).

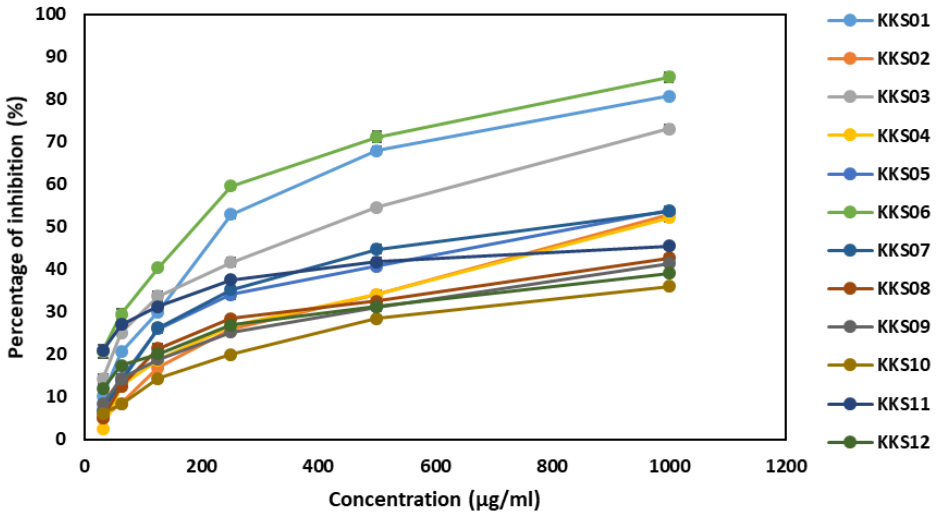

Figure 4. Percentage of inhibition (DPPH ASSAY).

Anticancer activity against HeLa cell line

To combat the problem of drug resistance and the negative side effects linked to several proven synthetic anticancer treatments, the search for new anticancer compounds from natural sources is essential. The remarkable capacity of marine sponge derivatives to impede the spread of malignancies has led to intensified research activities aimed at discovering innovative anticancer medicines. (Zhang et al. 2017; Ćetković et al. 2018). In terms of the variety of their bioactive metabolites, marine sponges are a "gold mine," and they could produce future medicines for a number of serious, globally prevalent diseases (Koopmans et al. 2009).

HeLa cells were incubated with ethylacetate extract of KKS6which showed strong antioxidant activity among other metabolites for 48 hours to test whether growth could be inhibited at concentration between 512 to 1 µg/mL. Figure 5 represents the percentage of inhibition and minimum inhibitory of ethylacetate extract of KKS6 against HeLa cell line. The percentage inhibition was observed to be 35.28±0.1, 36.44±0.4, 37.84±0.3, 43.09±0.4, 55.14±0.8, 57.93±1.2, 70.03±2, 74.98±1.4, 85.51±3.9, 90.37±1.4 at concentration between 1 to 512 µg/mL respectively. Isolate KKS6 significantly inhibited growth of HeLa cell line, with IC_{50} values of 55.32±1.2 µg/mL. *Microbacterins* A and B, significantly inhibited the growth of human tumour cell lines such as Bel-7402, HCT-8, A549, A2780 and BGC 823 (Liu et al. 2015). Previous research has reported that the *B. velezensis* isolated from marine sediments hadanticancer properties against MCF-7 cell line (Mostafa et al. 2019). Lodopyridone, an alkaloid from a marine *Saccharomonospora* species, was discovered to be cytotoxic (IC50 = 3.6 M) to HCT-116 human colon cancer cells (Maloney et al. 2009). Hence from comparing the result with the current study, it was evident that crude metabolite extracted from *Spongia officinalis* bacteria can be a potent anticancer drug.

Biochemical characterization

The isolate KKS66 was grown in King's B medium and incubated at 37°C for 24 hours. Pure colony of isolate 6 (blue- green colonies) under UV light was depicted in Figure 6. Biochemical characterization of KKS6was carried out. Results revealed that KKS6 was all test except citrate and oxidase are negative as given in table 3. From the results it is evident the organism belongs to genus *Pseudomonas.*

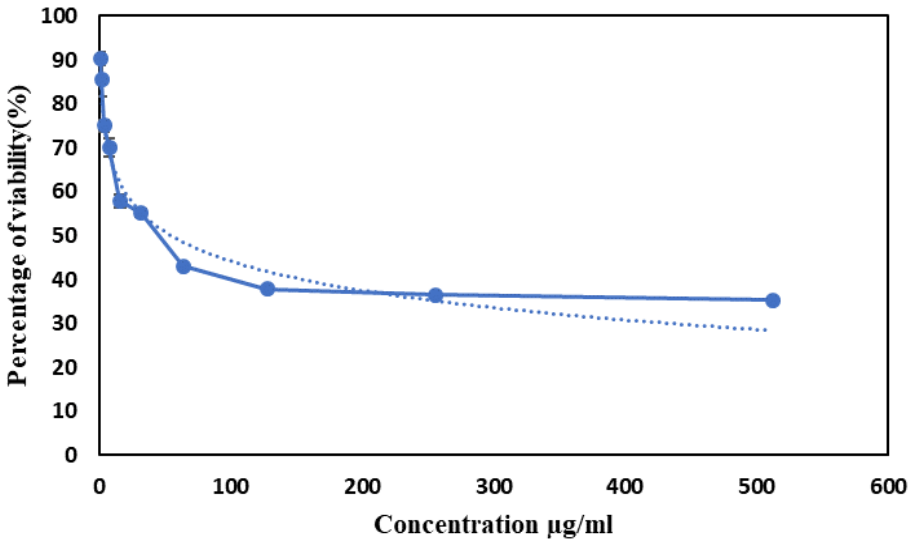

Figure 5. Percentage of viability (HeLa cell line).

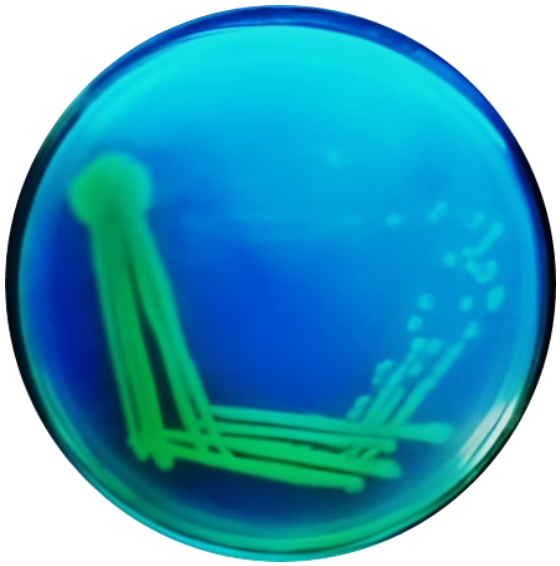

Figure 6. Pure colony isolation of KKS6.

Table 3. Biochemical characterization of KKS6.

Biochemical Test	Observation	Result
Indole Test	No pink or red color formation- The organism could not decompose tryptophane to indole	Negative
Methyl Red Test	No color change and hence no glucose fermentation	Negative
Voges Proskauer Test	No colour change and hence no production of acetylmethyl carbinol from glucose fermentation	Negative
Citrate Test	Change to blue color indicates the ability of organism to use citrate as sole carbon source	Positive
Tsi	Alkaline slant and bottom indicates no carbohydrate fermentation	Negative
Oxidase	The purple color change indicates the ability of organism to produce cytochrome c oxidase	Positive
Urease	No hydrolysis of urea indicate negative result	Negative

Molecular identification by 16s rRNA sequencing

DNA isolation was performed and PCR product was validated by agarose gel electrophoresis shown in Figure 7. The 16SrRNA partial sequence alignment database search of screened isolate shows 99% identity with *Pseudomonas fluorescens* strain by BLAST analysis. The phylogenetic tree was constructed with the aid of BLAST analysis (Figure 8). Hence the present study reveals that the query organism (KKS6) is identical to *Pseudomonas fluorescens* strain ATCC 13525.

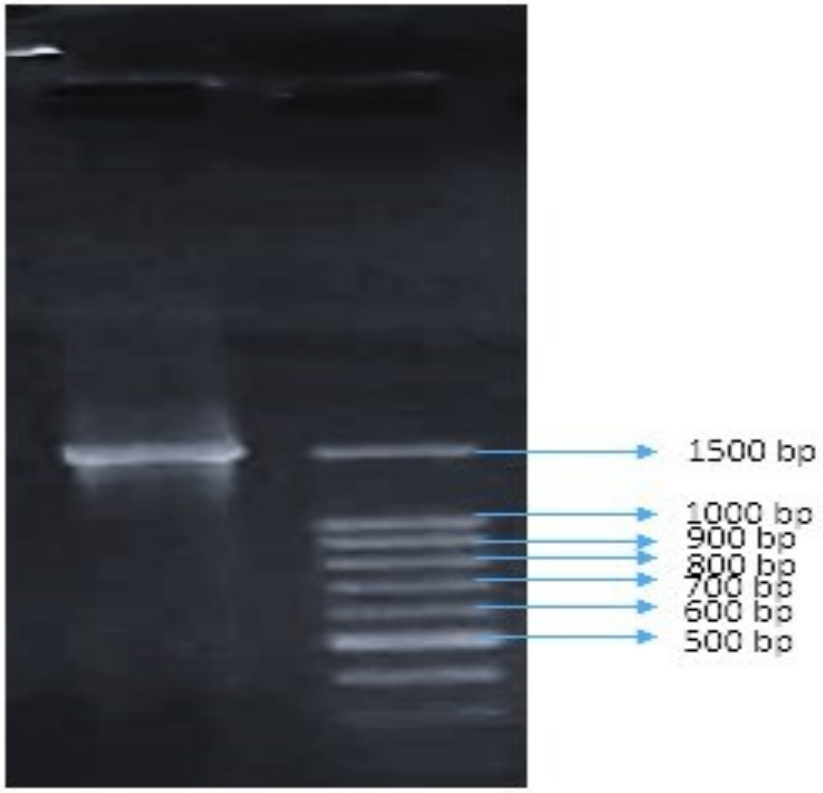

Figure 7. Agarose gel electrophoresis of PCR product (left- sample DNA: right -ladder.

CONCLUSION

In conclusion, the study identified 12 symbiont bacterial isolates from the marine sponge *Spongia officinalis*, with morphological characteristics re-

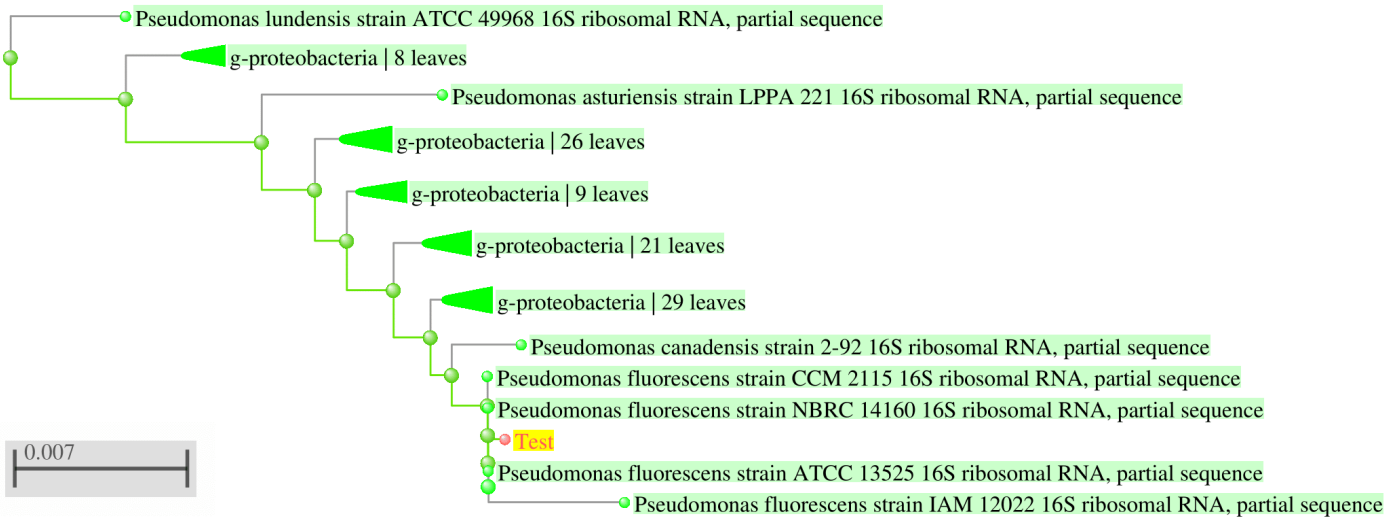

Figure 8. Phylogenetic tree (Neighbour – joining method- Kimura two-parameter (K2P) Model).

vealing predominant Gram-positive rods. Ethylacetate extracts exhibited diverse bioactive compounds, notably phenols and flavonoids. Isolate KKS6 showed remarkable antioxidant activity with an IC50 value of 167.26±0.1 µg/mL and significant inhibition of HeLa cell line growth with an IC50 value of 55.32±1.2 µg/mL compared to extracts. The 16s rRNA sequencing confirmed the organism to be *Pseudomonas fluorescens*. These findings suggest the potential of *Spongia officinalis*-associated symbiont bacteria, particularly *Pseudomonas fluorescens*, as a source of bioactive compounds with antioxidant and anticancer properties. The future study will focus on in-vivo studies to confirm the potential of these metabolites as potential drug candidate.

AUTHORS CONTRIBUTION

Conceptualization, M.J. Y.D. and U.S.; Data curation, M.J. and U.S.; Investigation, M.J. Y.D and U.S.; Supervision, M.J.; Validation, M.J. and Y.D.; Roles/Writing - original draft, M.J.; Writing - review & editing, M.J. and U.S.

ACKNOWLEDGMENTS

The authors extend their sincere gratitude to Simbioen Labs and Scientific Services Private Limited for their invaluable support, which enabled the research to be conducted seamlessly within their laboratory facilities.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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