

Cultivable Bacterial Symbionts from the Marine Sponge with Biological Activity

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ABSTRACT

Sponges serve as hosts for diverse organisms and hence are reservoirs of various natural products. The two symbiotic bacteria obtained from the marine sponge *Spongia officinalis* var. *ceylonensis* were identified as *Micrococcus* and *Planococcus* by morphological, biochemical, and molecular methods. The organic extract prepared from these sponge-associated bacteria was subjected to various bioactivity assays. The ethyl acetate extracts from the isolates were found to exhibit antioxidant and antiacetyl cholinesterase activity. These extracts exhibited antiproliferative action against the three cell lines tested with the lowest IC 50 value against the HT-29 cells. The GCMS analysis of these extracts revealed the presence of several bioactive compounds such as eugenol, phenol-2, 4-bis (1phenylethyl), methyl salicylate, 1, 2- benzenedicarboxylic acid esters, octadecanoic acid, squalene, and cannabinal. These bioactive compounds may be contributing to the activity of these symbiotic bacterial extracts and these symbiotic bacteria could serve to meet the supply problem in marine drug development.

Introduction

Marine sponges are envisaged as microbial diversity hotspots, as they harbor enormous diversity of microorganisms. Symbiotic microbes have recently been demonstrated as the site of biosynthesis of sponge-derived metabolites with pharmaceutical activity. The diversity of microorganisms inside these evolutionarily oldest animals covers most bacterial phyla. Sponges maintain a unique relationship with the microbes which constitutes up to 40-60% of their biomass [1]. These diverse microbes enter the sponge body through their filter-feeding habit, and some of them are ingested while others reside inside the sponge mesohyl as symbionts. These symbionts play significant roles in the nutrition, health, and chemical defense of sponges, and the symbionts, in turn, benefit from nutrients and protection by the sponge host [2]. Approximately 0.1% of the bacterial species are known until now [3] and approximately 32 bacterial phyla and candidate phyla have been found in sponges [4]. This clearly illustrates the great microbial diversity left unexplored which may serve as a good

source of bioactive compounds. The compounds from marine symbiotic microbes have been found to possess varied biological activities as several metabolites with antibacterial, antioxidant, immunosuppressive, antihypertensive, hypocholesterolemic, antiplasmodial, and anticancer activities are reported from them.

The marine sponge, *Spongia officinalis* var. *ceylonensis*, found exclusively in the Indian Ocean was found to possess significant pharmaceutical property [5,6]. This sponge belongs to the order Dictyoceratida which contributed the greatest number of compounds in the period 2001- 2010 [7]. The symbiotic microbes may be the producers or involved in the production of these metabolites in marine invertebrates. Some bacterial symbionts are host-specific and will be actively involved in the synthesis of species-specific compounds. Though the marine sponges are well known for their symbiotic microbial association, sponges of the Indian coast are not much explored beyond their bioactive potential. Here, we try to identify the symbiotic bacteria significantly contributing to the bioactivity of this sponge species.

Methodology

Sponge Collection and Isolation of Sponge-Associated Bacteria

The marine sponge *Spongia officinalis* var. *ceylonensis* was collected from the Southwest coast of India at a depth of 8-10 feet and was transferred to the laboratory in sterile plastic bags. The samples were identified with the help of Dr. P.A. Thomas, Retd. Principal Scientist, CMFRI, Vizhinjam. The collected marine sponge sample was washed thoroughly with artificial seawater followed by 70% alcohol. The treated sponge specimen was homogenized with sterilized mortar and pestle and was serially diluted. These serially diluted samples were spread plated and streaked onto agar plates containing the Zobell marine broth medium. The colonies with distinct morphology were re-streaked to obtain pure colonies. Of the 12 bacteria isolated, two isolates - MBTU SOLY1 and MBTU SOBOP1 that showed greater cytotoxicity in brine shrimp lethality assay were chosen for further studies. The selected strains were preserved in 20-30% glycerol at -20°C.

Morphological, Biochemical and Physiological Characteristics of the Symbiotic Bacteria

The pure colonies of the two selected isolates were carefully observed for identifying their morphology. Standard protocols were used for determining motility, gram staining, and various biochemical characteristics such as catalase and oxidase activities, nitrate reduction, etc. An extensive range of biochemical properties was analyzed by using the VITEX2 system. Physiological characterization of the two strains was done after adjusting the absorbance of the broth cultures to 10D and various parameters such as different concentrations of NaCl, various temperatures, pH, and varying seawater concentrations were evaluated.

Phylogenetic Identification of the Isolates

The two isolates were identified molecularly by employing the primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' AAGGAGGTGWTCCARCC 3') for the amplification of 16S rRNA. PCR was carried out as described by Chun and Goodfellow [8]. The amplification program consisted of an initial denaturation step of 94°C for 3 min, and 30 cycles of reaction with each cycle of 94°C for 30s, 58°C for 30s, 72°C for 2min, and a final extension step of 72°C for 7minutes. PCR product was checked by agarose gel electrophoresis, amplified DNA fragments were observed and sequencing was performed. The unknown bacterium was identified using the Genbank database. The obtained sequences were analyzed using the BLAST tool to get the relative identification of each bacterial species [9] and the culture sequences were deposited in Genbank through NCBI and obtained accession numbers.

Preparation of Microbial Extract

The isolates were inoculated into sterile Zobell marine broth

and kept at 25°C for 3 days. Turbidity was seen during the incubation period. 1mL (~4%) of inoculum of three-day-old cultures were transferred to 250mL marine broth and kept at 25°C for 7 days. The cultures were then centrifuged at 10000 rpm for 30 minutes and the supernatant was collected. The collected supernatants were lyophilized and the solvent ethyl acetate was added in the ratio 1:1 to the supernatant taken in a separating funnel and extracted for three days. The extracts were then concentrated by evaporating the solvent in a rotary evaporator. The dried extracts were stored at -20°C.

Screening of Chemical Constituents

The ethyl acetate extracts of the symbiotic microbial isolates were screened for chemical constituents with slight modifications of the method (Table 1) of Harborne [10].

Table 1: Methods for screening for chemical constituents.

Sl No.	Test	Positive Reaction
1.	Test for steroids: Extract taken in acetic acid. Warmed and cooled it under tap water and H ₂ SO ₄ was dropped sidewise.	Green color
2.	Test for terpenoids: To the cold extract chloroform was added and con. H ₂ SO ₄ was dropped sidewise.	Yellow layer in the acid layer
3.	Test for reducing sugar: To the cold extract, Molisch's reagent was added.	Purple color.
4.	Test for alkaloids: The cold extract was mixed with acetic acid (aqueous layer decanted) and followed by the addition of a few drops of Dragendoff's reagent.	An orange/red-orange precipitate
5.	Test for phenolics: To the cold extract, neutral FeCl ₃ was added.	An intense blue/violet color
6.	Test for xanthoprotein: To the cold extract, 1N Con.HNO ₃ was added followed by excess ammonia	Red-orange precipitate
7.	Test for aromatic acids: To the cold extract, saturated NaHCO ₃ was added.	Brisk effervescence
	Test for flavonoids: 1-2mg of Mg ²⁺ was added to the cold extract, followed by 0.5mL of the con. HCl	Red/orange color

Antioxidant Activity Assay

The organic extracts of the symbiotic microbial extracts were subjected to the following antioxidant assays.

DPPH Radical Scavenging Activity Assay

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the microbial extracts was determined using the method of Mensor et al. [11]. The samples and the reference at differing concentrations of 62.5µg/mL, 125µg/mL, 250µg/mL, 500µg/mL and 1000µg/mL were dissolved in methanol were then mixed with the DPPH solution. The remaining DPPH amount was measured at 517nm using a spectrophotometer. Ascorbic acid was employed as the reference standard. Inhibition of DPPH in percentage was calculated as given below:

$$I\% = \left[\frac{(A_b - A_s)}{A_s} \right] \times 100$$

where A_b is the absorbance of the control reaction and A_s is the absorbance of the extracts.

Nitric Oxide Scavenging Assay

This assay was performed according to the protocol of Rana et al. [12]. 10mM Sodium nitroprusside (2mL) in phosphate buffer saline was incubated with the test compounds at different concentrations for 30 minutes at room temperature. 0.5mL of the incubated solution was then mixed with mL of Griess reagent and the absorbance was measured at 546nm. Ascorbic acid was used as the reference standard. The radical scavenging activity was calculated as per the equation:

$$\%inhibition = \left[\frac{(A_0 - A_1)}{A_1} \right] \times 100$$

where A_0 is the blank and A_1 is the test sample.

Acetylcholinesterase Inhibition Assay

The acetylcholinesterase inhibition activity was performed for the extracts of the sponge symbiotic bacteria at varying concentrations according to the method of Ellman et al. [13], with slight modifications. Acetylcholinesterase (AChE) from electric eel (Sigma, USA) was dissolved in 100mM phosphate buffer (pH 7.3) to a final concentration of 500EU/mL. 140 μ L of the Ellman reagent (5, 5-dithiobis-2-nitrobenzoic acid) in 25 mM phosphate buffer (pH 7.0) is added to each microtiter plate wells followed by the addition of 10 μ L acetylcholine (ACh) in 1mM final concentration, 20 μ L of sponge sample, and 50 μ L of AChE. Deionized (20 μ L) water was used as controls. Galantamine was used as the reference standard. The time course of the enzymatic reaction was monitored for 12 minutes at 412nm at 25°C.

Antiproliferative Activity

The ethyl acetate extracts of the two isolates were subjected to MTT assay against HT-29 colon cancer cell line, MCF-7 breast cancer cell line, and HeLa cervical cancer cell lines purchased from NCCS Pune. These cells were maintained in Dulbecco's Modified Eagles Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and grown to confluency at 37°C in 5% CO₂ in a humidified atmosphere in a CO₂ incubator. The trypsinized (500 μ L of 0.025% Trypsin in PBS or 0.5mM EDTA solution for two minutes) cells were passaged to T flasks under aseptic conditions. The cells were then treated with varying concentrations (6.25 μ g/mL, 12.5 μ g/mL, 25 μ g/mL, 50 μ g/mL and 100 μ g/mL) of the extract from a stock of 1mg/mL. The percentage viability was determined by standard MTT assay after 24 hours of incubation. 30 μ L of MTT solution (5mg/mL in PBS) was then added to the treated cells after 1X PBS wash. It was then incubated at 37°C for 3-5 hours. MTT was removed by washing with 1X PBS and 200 μ L of DMSO was added to

the culture and incubated for half an hour. Absorbance was read at 540 nm employing DMSO as blank in a microplate reader.

$$\%inhibition = \left[\frac{(A_c - A_t)}{A_c} \right] \times 100$$

where A_c is OD of Control and A_t is OD of Test.

Statistical Analysis

The results of the experiments done in triplicates were analyzed by One way ANOVA and were considered statistically significant when $p < 0.05$. Error bars in graphs represent standard error of means per triplicate samples and IC 50 values were calculated in MS Excel. The values were compared by Tukey's post hoc test and the superscript on the table represents the values that differ significantly (** $P < 0.0001$, ** $P < 0.001$, * $P < 0.005$).

GC-MS Analysis

The ethyl acetate extracts of the two isolates were subjected to GCMS analysis. Analyses were carried out on an Agilent GC 7890A MS575C fitted with DB-SMS 30x0.25mmx0.25 μ m using Helium as the carrier gas. The injective temperature was set at 250°C. The column temperature was initially kept at 40°C for 5 min, then gradually increased to 250°C at a rate of 5°C/min and finally raised to 280°C hold for 10 minutes. The identification of the compounds was done with the help of the NIST library.

Results

Morphological, Biochemical and Physiological Characteristics of the Symbiotic Bacteria

The two isolates- MBTU SOLY1 and MBTU SOBOP1, obtained from the sponge host *Spongia officinalis* var *ceylonensis* were identified by observing their culture characteristics, microscopic observation, biochemical identification, and molecular identification. MBTU SOLY1 was a yellow, small, entire, opaque, round, and smooth colony whereas MBTU SOBOP1 was an orange, small, round, entire, flat, and smooth colony. Both the isolates were gram- positive, non-motile cocci. The biochemical characterization of the isolates obtained by both manual and VITEK 2 system is summarized in Table 2. The results indicated that both the isolates were positive for catalase, oxidase, and alpha-glucosidase, possessed arylamidase specific for leucine and tyrosine, and capable of growth in 6.5% NaCl. The isolate MBTU SOLY1 was urease positive, arginine dihydrolase positive (can utilize arginine as a substrate), and possessed arylamidases for Ala-Phe-Pro, L-Proline, L-pyrrolidonyl, and alanine whereas it was incapable of sugar fermentation. The isolate MBTU SOBOP1 was optochin resistant, possessed arylamidase for L-aspartate was positive for alpha-galactosidase and beta- galactosidase, N-acetyl -D- glucosamine, and could ferment the carbohydrates-D- xylose, D-galactose, D-ribose, D-raffinose, D-mannose, saccharose/sucrose, D-trehalose, D- mannitol, salicin, and D-sorbitol.

Table 2: Biochemical characterization of the two isolates.

Test	MBTU SOLY1	MBTU SOBOP1
Indole production Test	-	-
Methyl red test	-	-
Voges –Proskauer test	-	-
Nitrate reduction test	-	-
Citrate utilization test	-	-
Urease test	+	-
Catalase Test	+	+
Oxidase Test	+	+
D-AMYDGALIN	-	-
CYCLODEXTRIN	-	-
D-XYLOSE	-	+
D-GALACTOSE	-	+
D-RIBOSE	-	+
LACTOSE	-	-
PULLAN	-	-
D-RAFFINOSE	-	+
D-MANNOSE	-	+
D-MALTOSE	-	-
SACCHAROSE/SUCROSE	-	+
D-TREHALOSE	-	+
D-MANNITOL	-	+
SALICIN	-	+
D-SORBITOL	-	-
PHOSPHATIDYLINOSITOL PHOSPHOLIPASE C	-	-
ARGININEDIHYDROLASE 1	+	-
BETA-GALACTOSIDASE	-	+
ALPHA-GLUCOSIDASE	+	+
Ala-Phe-Pro ARYLAMIDASE	+	-
L-Aspartate ARYLAMIDASE	-	+
BETA GALACTOPYRANOSIDASE	-	-
ALPHA-MANNOSIDASE	-	-
PHOSPHATASE	-	-
Leucine –ARYLAMIDASE	+	+
L-Proline ARYLAMIDASE	+	-
BETA –GLUCURONIDASE	-	-
ALPHA-GALACTOSIDASE	-	+
L-Pyrrolidonyl- ARYLAMIDASE	+	-
BETA-GLUCURONIDASE	-	-
Alanine ARYLAMIDASE	+	-
Tyrosine ARYLAMIDASE	+	+
ETHYL-B-D-GLUCOPYRANOSIDE	-	-
N-ACETYL-D-GLUCOSAMINE	-	+
ARGININE DIHYDOLASE 2	-	-
L-LACTATE alkanisation	-	-
GROWTH IN 6.5% NaCl	+	+

POLYMYXIN B RESISTANCE	-	-
BACITRACIN RESISTANCE	-	-
NOVOBIOCIN RESISTANCE	-	-
OPTOCHIN RESISTANCE	-	+
O12 RESISTANCE (comp. vibrio)	-	-

The physiological characterization of the isolates revealed that the optimum temperature for both the cultures was at 25°C. They were capable of surviving till 55°C which indicates the thermophilic nature of the isolates, but the growth was found to decrease with temperature. The optimum pH was 8 for both the cultures, but the growth of both the isolates got inhibited at a lower pH range. The optimum NaCl concentration was 7% for MBTU SOLY1 and 4% for MBTU SOBOP1 and these isolates could survive up to 30% NaCl concentration. The Zobell medium in 50% seawater and 2% NaCl concentration was found to be the suitable medium for the optimum growth of the selected isolates on monitoring the growth

of these isolates in different media such as Zobell media in 50% seawater, Zobell media in 100% seawater, Zobell media in distilled water, Zobell media in 50% seawater and 2% NaCl concentration Zobell media in distilled water and 2% NaCl concentration.

Phylogenetic Identification of the Isolates

The sequencing results confirmed that MBTU SOLY1 belonged to *Micrococcus luteus* sp. (Figure 1) and MBTU SOBOP1 belonged to *Planococcus* sp. (Figure 2). Both the sequences were deposited in Genbank through NCBI and obtained accession numbers as KT734853 and KX236450 respectively.

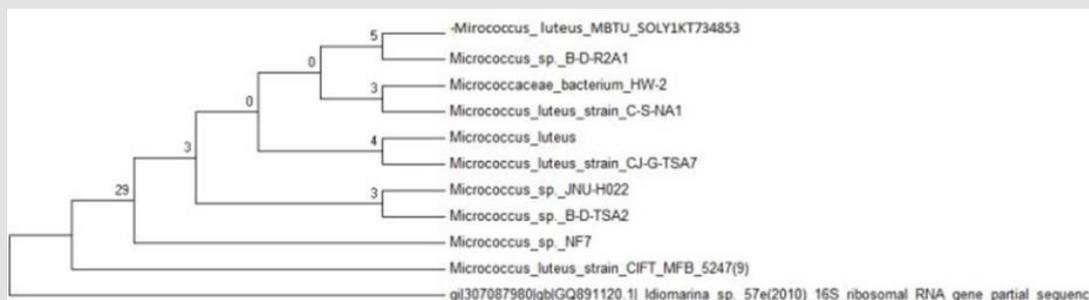


Figure 1: Phylogenetic tree of MBTU SOLY1.



Figure 2: Phylogenetic tree of MBTU SOBOP1.

Screening of Chemical Constituents

The supernatants of both the isolates were lyophilized and were subjected to extraction using the solvent ethyl acetate. The chemical constituent analysis (Table 3) revealed the presence of

similar chemical entities in both the extracts such as triterpenoids, phenolics, reducing sugar, aromatic acid, and steroid. These chemicals are potent bioactive entities that may contribute to the bioactivity of the extracts.

Table 3: Screening of chemical constituents in microbial extracts- '+' indicates the presence and '-' indicates an absence of the chemical constituent in the extract.

Sample	Alkaloid	Xanthoprotein	Triterpenoid	Phenolics	Reducing sugar	Aromatic acid	Steroid	Flavanoid
MBTU SOLY1 extract	-	-	+	+	+	+	+	-
MBTU SOBOP1 extract	-	-	+	+	+	+	+	-

Antioxidant Activity

Antioxidants find major application in ailments such as cancer, aging, and atherosclerosis due to the involvement of free radicals in disease development. The extracts of both the isolates exhibited significant antioxidant activity. DPPH radical scavenging activity assay was performed for different concentrations of both the extracts of the two isolates at 62.5µg/mL, 125µg/mL, 250µg/

mL, 500µg/mL, and 1000µg/mL concentrations to test the free radical scavenging ability of these extracts. The DPPH free radical is reduced to yellow diphenylpicrylhydrazine at all the tested concentrations. The extracts exhibited scavenging activity in a concentration-dependent manner. The inhibition percentage ranges from 9.4-80.06% and the extract of MBTU-SOLY1 exhibited greater activity (Figure 3).

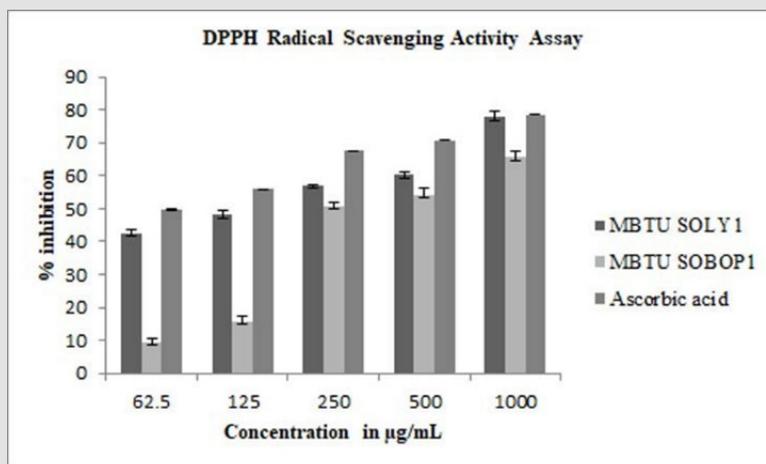


Figure 3: DPPH radical scavenging activity of microbial extracts- Error bars in graphs represent standard error of means per triplicate samples.

Nitric oxide is a bioregulatory molecule and exerts various physiological effects including blood pressure, neuronal signal transduction, platelet function, antimicrobial and antitumor activity. Increased nitric oxide production is a sign of inflammation and infection causes renal dysfunction and tumor growth. Both

the extracts possessed NO scavenging activity and the extract of MBTU SOLY1 exhibited greater activity with an IC 50 value of 78.35µg (Table 4). Both the extracts exhibited elevated nitric oxide scavenging activity in a dose-dependent manner (Figure 4).

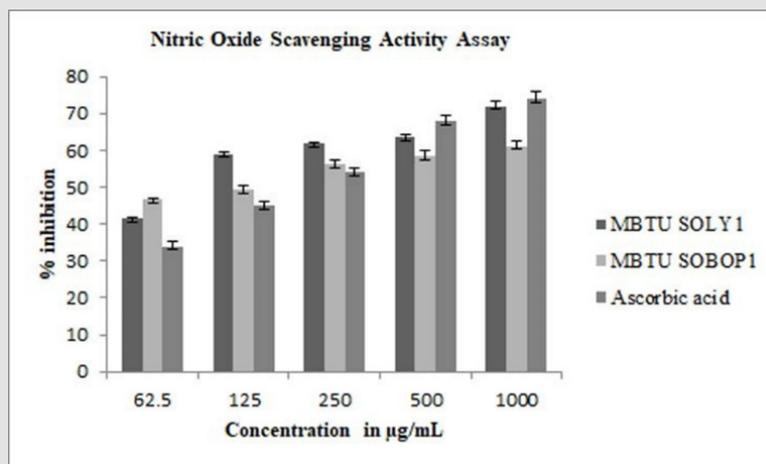


Figure 4: Nitric oxide scavenging activity of microbial extracts- Error bars in graphs represent standard error of means per triplicate samples.

Table 4: IC₅₀ values of antioxidant assays- The superscript on the table represents the values that differ significantly (**P<0.0001, **P< 0.001, *P < 0.005).

	MBTU SOLY1	MBTU SOBOP1	Ascorbic acid
DPPH radical scavenging activity assay	120± 1.23***	240± 0.98***	53 ± 1.13
Nitric oxide scavenging assay	78.35±1.12**	122± 0.54**	180 ± 1.23

Acetylcholinesterase Inhibitory Activity

Anticholinesterases cause the accumulation of acetylcholine at their sites of action thus stimulating the parasympathetic nervous

system-slowing heart action, lowering blood pressure, increasing secretion, and inducing contraction of smooth muscles. In this study, AChE inhibitory activity (Figure 5) was greater for MBTU SOLY1 extracts with an IC₅₀ value of 530µg (Table 5).

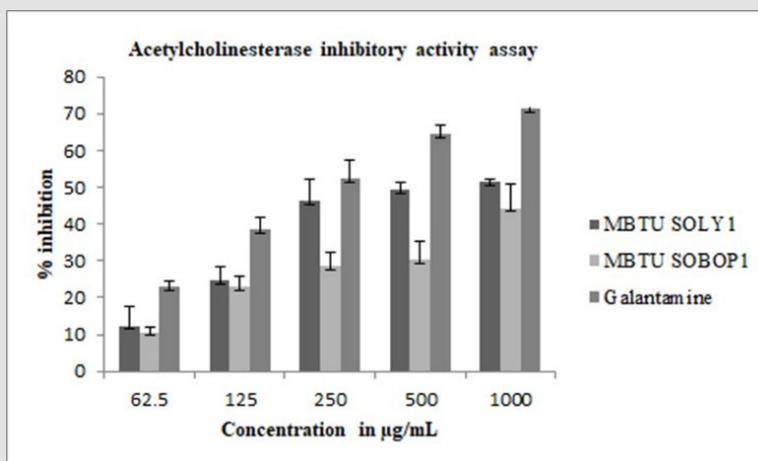


Figure 5: Acetylcholinesterase inhibitory activity of microbial extracts- Error bars in graphs represent standard error of means per triplicate samples.

Table 5: IC₅₀ values of AChE inhibitory activity assay- The superscript on the table represents the values that differ significantly (**P<0.001, **P<0.001, *P<0.005).

	MBTU SOLY1	MBTU SOBOP1	Galantamine
IC ₅₀ values in µg/mL	530.34± 1.24**	948.77± 0.98***	246.71± 0.58

Antiproliferative Activity

MTT assay for ethyl acetate extracts of microbes was performed against three cancer cell lines- HT-29, MCF-7, and HeLa cell lines. Their activities were concentration-dependent and the viability of the cells decreased with the increasing concentrations of both the

extracts. Against all the three tested cell lines, the extract of MBTU SOLY1 was more active (Figure 6 and Table 6). Among these three cell lines, the microbial extracts were more active against the HT-29 cell line with an inhibition range of 61-79% for MBTU SOLY1 extract and 31-73% for MBTU SOBOP1 extract.

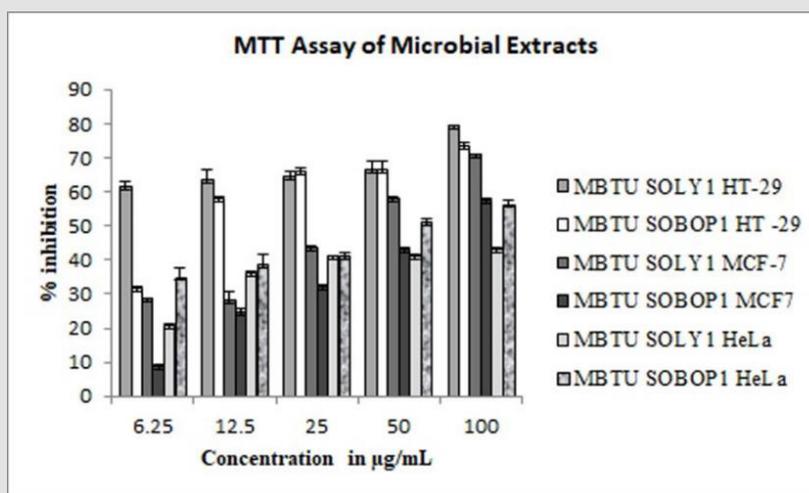


Figure 6: MTT assay of microbial extracts- Error bars in graphs represent standard error of means per triplicate samples.

Table 6: IC₅₀ values of MTT assay- The superscript on the table represents the values that differ significantly (**P<0.001, *P < 0.005).

	MBTU- SOLY1 HT- 29	MBTU- SOLY1 MCF-7	MBTU- SOLY1 HeLa	MBTU SOBOP1 HT-29	MBTU SOBOP1 MCF-7	MBTU SOBOP1 HeLa
IC ₅₀ Values in µg/ mL	36 ± 1.33**	45 ± 0.78**	73 ± 1.45**	51±1.67**	56 ± 1.24**	68 ± 1.77**

GCMS Analysis

The two bioactive ethyl acetate extracts were analyzed by GCMS. The result indicated that both the extracts contained peaks of the compounds eugenol, phenol-2,4-bis(1phenylethyl), methylsalicylate, 1,2-benzenedicarboxylic acid esters, fatty acid esters, squalene, and cannabinol (Figures 7&8; Tables 7&8). All

these bioactive compounds such as phenol-2,4- bis(1phenylethyl), 1,2-benzenedicarboxylic acid esters, and fatty acid esters were also present in the host sponge extract (Supplementary material). These compounds are reported to have antibacterial, antitumor, antioxidant activities (Table 9). This proves the symbiotic association of sponge with these microbes.

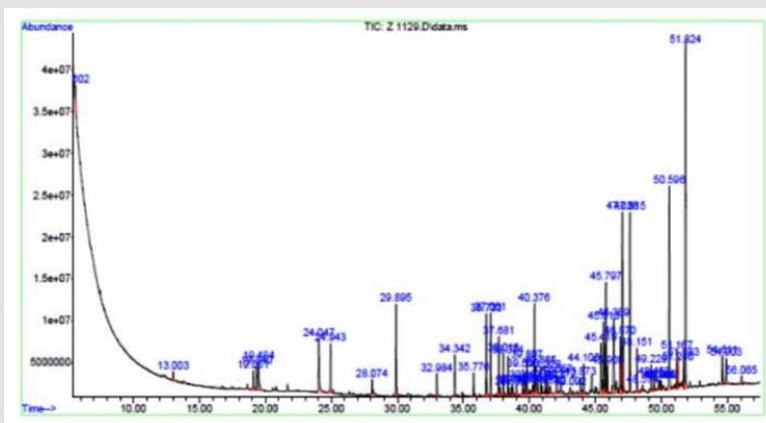


Figure 7: GCMS profile of MBTU SOLY1 extract.

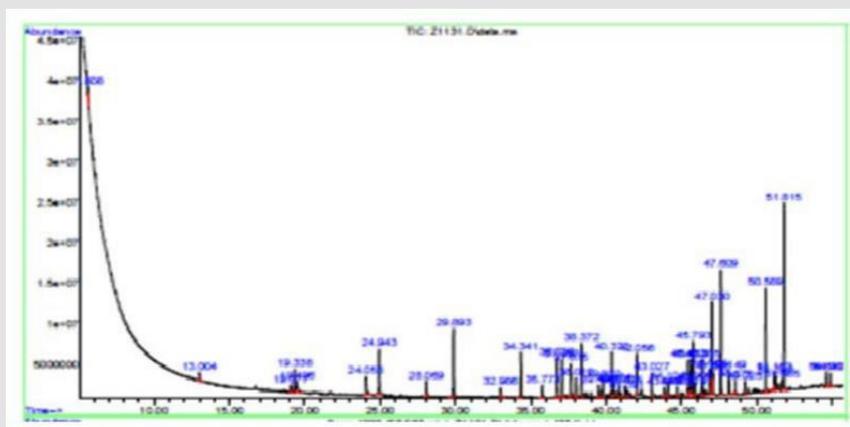


Figure 8: GCMS analysis of MBTU SOBOP1 extract.

Table 7: Compounds in the ethyl acetate extract of the isolate MBTU SOLY1 identified through GCMS.

Compounds in ethyl acetate extract of MBTU SOLY1	Retention time	Molecular Formula
Ethane, 1,1-diethoxy	5.60	C ₆ H ₁₄ O ₂
1-Decene	13.00	C ₁₀ H ₂₀
Cyclohexanol, 1-methyl-4-(1-methylethyl)	19.08	C ₁₀ H ₂₀ O
1-Dodecene	19.34	C ₁₂ H ₂₄
Methyl salicylate	19.48	C ₈ H ₈ O ₃

Eugenol	24.04	$C_{10}H_{12}O_2$
1-Tetradecene	24.94	$C_{14}H_{28}$
Phenol, 2,4-bis(1,1-dimethylethyl)	28.07	$C_{14}H_{22}O$
Phenol, 2-(1-phenylethyl)	32.98	$C_{14}H_{14}O$
3-Octadecene, (E)	34.34	$C_{18}H_{36}$
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	35.77	$C_{16}H_{22}O_4$
4-(2-Oxopropyl)-6-bromo-2H-1,4-benzoxazin-3-one	36.73	$C_8H_6BrNO_2$
Hexadecanoic acid, methyl ester	37.06	$C_{17}H_{34}O_2$
1-Nonadecene	38.37, 42.05	$C_{19}H_{38}$
9,12-Octadecadienoic acid (Z, Z)-, methyl ester	40.24	$C_{19}H_{34}O_2$
11-Octadecenoic acid, methyl ester	40.37	$C_{19}H_{36}O_2$
Octadecanoic acid, methyl ester	40.86	$C_{19}H_{38}O_2$
1,2-Benzenedicarboxylic acid, dipentyl ester	41.22	$C_{18}H_{24}O_4$
N,N-Dimethyldodecanamide	43.09	$C_{14}H_{29}NO$
1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester	43.87, 45.61	$C_{22}H_{34}O_4$
Phenol, 2,4-bis(1-phenylethyl)	45.09, 46.87	$C_{24}H_{26}O$
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	47.61	$C_{16}H_{22}O_4$
1-Docosene	48.57	$C_{22}H_{44}$
1,2-Benzenedicarboxylic acid, diisodecyl ester	51.16, 51.24, 51.81	$C_{28}H_{46}O_4$
Squalene	51.68	$C_{30}H_{50}$
Cannabinol, trifluoroacetate	54.61, 54.90	$C_{23}H_{25}F_3O_3$

Table 8: Compounds in the ethyl acetate extract of the isolate MBTU SOLY1 identified through GCMS.

Compounds in ethyl acetate extract of MBTU SOBOP1	Retention time	Molecular Formula
Ethane, 1,1-diethoxy	5.61	$C_6H_{14}O_2$
1-Decene	13.00	$C_{10}H_{20}$
Menthol	19.08	$C_{10}H_{20}O$
1-Dodecene	19.34	$C_{12}H_{24}$
Methyl salicylate	19.48	$C_8H_8O_3$
Eugenol	24.04	$C_{10}H_{12}O_2$
1-Tetradecene	24.94	$C_{14}H_{28}$
1-Hexadecanol	29.89	$C_{16}H_{34}O$
Phenol, 2-(1-phenylethyl)	32.98	$C_{14}H_{14}O$
E-15-Heptadecenal	34.34	$C_{18}H_{36}$
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	35.77	$C_{16}H_{22}O_4$
Hexadecanoic acid, methyl ester	37.05	$C_{17}H_{34}O_2$
1-Nonadecene	38.37	$C_{19}H_{38}$
9-Octadecenoic acid, methyl ester, (E)	40.37	$C_{19}H_{36}O_2$
Octadecanoic acid, methyl ester	40.86	$C_{19}H_{38}O_2$
1,2-Benzenedicarboxylic acid, dipentyl ester	41.22	$C_{18}H_{24}O_4$
1-Docosene	42.05, 48.57	$C_{22}H_{44}$
Tributyl acetyl citrate	43.02	$C_{20}H_{34}O_8$
1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester	43.86, 45.61	$C_{22}H_{34}O_4$
Phenol, 2,4-bis(1-phenylethyl)	45.90, 46.86	$C_{24}H_{26}O$
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	47.60	$C_{16}H_{22}O_4$
1,2-Benzenedicarboxylic acid, diisodecyl ester	48.14, 51.16, 51.24, 51.81	$C_{28}H_{46}O_4$
Squalene	51.68	$C_{30}H_{50}$
Cannabinol, trifluoroacetate	54.61, 54.90	$C_{23}H_{25}F_3O_3$

Table 9: The activity of chemicals in the extract.

Compound	Activity
Hexadecanoic acid, methyl ester	Antibacterial, antifungal [38]
9,12-Octadecadienoic acid (Z,Z) methyl ester	Anticancer [39]
1,2-benzenedicarboxylic acid, diisooctyl ester	Antimicrobial, antifouling [40]
1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	Cytotoxic [36]
Squalene	Antibacterial, antioxidant, antitumor, cancer- preventive, chemopreventive, immunostimulant, lipoxygenase inhibitor, pesticide [40]
Eugenol	Antimicrobial [41]
Phenol, 2,4-bis(1-phenylethyl)	Antioxidant, anti-inflammatory, cytotoxic, insecticide, nematocidal, antibacterial, antiviral, antifungal, autotoxic, allelopathic [42]
Methyl salicylate	Antimicrobial, antioxidant [43]
Tributyl acetyl citrate	Anticancer, antimicrobial [44]
Menthol	Antibacterial [45]

Discussion

Marine sponges are well-known hosts for diverse microbes. These microbes produce various secondary metabolites with the prospect of developing into useful drugs. Numerous compounds have been reported from marine sponges and many of them are believed to be the products of symbiotic microbes in them. In this study, the two gram-positive halophiles-MBTU SOLY1 and MBTU SOBOP1 isolated from the marine sponge *Spongia officinalis* var. *ceylonensis* were identified as *Micrococcus luteus* and *Planococcus* sp. respectively. This is the first report of isolating *Micrococcus* and *Planococcus* from the marine sponge *Spongia officinalis* var. *ceylonensis* from the Indian coast. The *Micrococcus* was isolated from various other marine sponges [14-17] and the *Planococcus* sp. was isolated from the marine sponge, *Plakortis simplex* [18]. Li [19] reviewed that the *Micrococcus* sp. has been previously isolated from *Hymeniacon perleve*, *Mycale adherens*, and *Callyspongia* sp. whereas the *Planococcus* sp. was isolated from *Craniella australiensis*. The isolate MBTU SOLY1 belonged to Phylum Actinobacteria and the isolate MBTUSOBOP1 belonged to Phylum Firmicutes. Thomas *et al* [17] have reported that the Phylum Actinobacteria is the most abundant in terms of the distribution of compound production in sponge symbionts which is followed by the Phylum Firmicutes. In an approach to isolate bacteria from *Spongia officinalis*, Karimi *et al* [20] revealed that the isolates belonged to the phyla Proteobacteria, Actinobacteria, and Firmicutes.

The chemical screening of organic extracts of the isolates revealed the presence of triterpenoids, phenolics, reducing sugar,

aromatic acid, and steroid in them which may be the contributors to the bioactive property of the extracts. Phytochemical characterization of metabolites of ethyl acetate extracts of *Pseudomonas* sp. from *Callyspongia* sp. showed that it contained alkaloids, quinones, flavonyl glycosides, and flavonoids [21]. In the same study, the extract from *Bacillus* sp. from *Haliclona* sp. was reported to contain carbohydrates, proteins, lipids, alkaloids, quinones, and flavonyl glycosides. The bioactive ethyl acetate extract of *Bacillus* sp. from the marine sponge *Petrosia* sp. from Bira Island, Jakarta, Indonesia contained flavonoids, steroids, and tannins [22]. Cita, *et al.* [23] reported the presence of alkaloids, steroids, and phenols in the ethyl acetate extract of the bacteria from the marine sponge *Xestospongia testudinaria*. In this study, both the extracts were found to possess significant antioxidant activity. The antioxidant activity of the extracts may be mainly due to the presence of the electron-donating nature of the bioactive chemical constituents present in them. Antioxidant activity of various sponge-associated bacteria was reported by various researchers [24-26]. Kim, *et al.* [27] evaluated the antioxidant activity of ethyl acetate extracts of 21 different marine bacteria isolated from floats, marine algae, animals, and sponges from Jeju Island, Korea.

Acetylcholinesterase has a role in cell growth, signaling, and tumorigenesis which evokes the potential for anticancer drug development. The ability of the extracts to accumulate acetylcholine in the synapses gives an additional prospect for developing a drug molecule for neurodegenerative disease. The anticholinesterases in clinical use today are mainly for treating neurodegenerative diseases such as Alzheimer's disease and for augmenting gastric and intestinal contractions and muscular contractions in general and most of them are with side effects. The extracts in the present study exhibited an anticholinesterase effect which holds the prospect for the development of a novel drug candidate. *Bacillus subtilis* strain from the marine sponge, *Fasciospongia cavernosa* is reported to have acetylcholinesterase inhibitory activity [28]. The microbial extracts also exhibited concentration-dependent activity against the cancer cell lines and were found to be more active against the HT-29 colon cancer cell line. There are various reports for anticancer activities of extracts of marine sponge-associated bacteria [29,30].

The GCMS analysis of the extracts revealed the presence of several bioactive compounds such as eugenol, phenol-2,4-bis(1-phenylethyl), methylsalicylate, 1,2-benzenedicarboxylic acid esters, eugenol, fatty acid esters, squalene, and cannabinal in them. Some of them were common in both the extracts and were also found in the host sponge extract which proves the symbiotic association of these microbes. The compound menthol was only present in the MBTU SOBOP1 extract and not in the MBTU SOLY1 extract. This compound from plant extract is reported to have antibacterial and antioxidant activities by Singh, *et al.* [31]. There were several

reports on utilizing GCMS as a tool for discovering compounds in the extracts of marine bacteria [32-34]. Selvin et al. [35] observed the presence of 1,2 benzenedicarboxylic acid in the GCMS spectrum of ethyl acetate fraction of *Nocardioopsis darsonvillei* isolated from the sponge *Dendrilla nigra*. Krishnan, et al. [36] reported that the compound 1,2 benzenedicarboxylic acid, mono (2-ethylhexyl) ester from the marine *Streptomyces* sp. VITSJK8 exhibited a cytotoxic effect against HepG2 and MCF-7 cell lines. Two non-motile gram-negative bacteria of Verrucomicrobia namely *Rubritalea spongiae* sp. nov. and *Rubritalea tangerine* sp. nov. from marine sponges were reported to produce the bioactive triterpene, squalene whose primary source is liver oil from deep-sea sharks and whales [37].

The symbiotic microbes produce these bioactive compounds inside the sponges as it is in the favorable physical environment for the induction of its secondary metabolism. But, the metabolic profile of these microbes changes rapidly once they are devoid of the selective pressure offered by the natural habitat. The successful culture of symbiotic microbes producing bioactive metabolites is of great significance since it will serve as an unlimited source of the respective compound while proving its contribution to the sponge host. Among the two isolates and their extracts, the MBTU SOLY1 ethyl acetate extract was more active in all the bioactivity assays. Though both the tested extracts contained similar chemical constituents, the greater activity of the MBTU SOLY1 extract may be

due to the higher concentration of the active principle. The study provides a clear idea with respect to the symbiotic association of the host marine sponge with the bacteria. The significant contribution of the symbionts towards the activity of the host marine sponge enables the economic development of the active compound as it is possible to cultivate the microbe in larger quantities for harvesting the active component in sufficient amounts. Thus, further optimization, bioassay- guided purification, and characterization studies of these isolates will yield potential drug candidates [38-45].

Acknowledgement

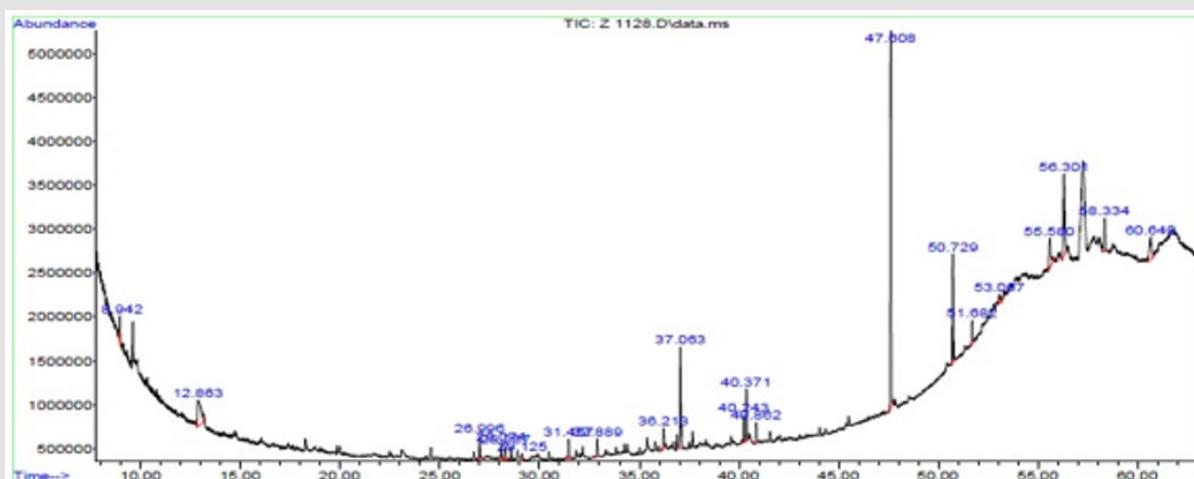
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Conflict of Interest

The authors declare that there is no conflict of interest.

Availability of Data and Material

The sequence data were submitted to GenBank through NCBI and obtained accession numbers KT734853 and KX236450. All data generated or analyzed during this study are included in this published article (and supplementary files).



Supplementary Figure1: GC-MS Profile of organic extract of marine sponge, *Spongia officinalis* var *ceylonensis*.

Supplementary Table1: Compounds present in the extract of *Spongia officinalis* var *ceylonensis* identified through GCMS.

Retention Time	Compound
8.94	Diethylphenol,tert-butyl dimethylsilylether
12.86	Decane
27	Dodecane-1-chloro
28.07	Phenol,2,4-bis(1,1-dimethylethyl)-
28.28	Methyl-10-methyl-undecanoate
29.12	Decane,1-bromo
31.46	Dodecane, 1-iodo-

32.88	Methyl tetradecanoate
36.21	Hexadecen-1-ol, trans-9
37.06	Hexadecanoic acid, methyl ester
40.24	8,11-Octadecadienoic acid, methyl ester
40.37	10-Octadecenoic acid, methyl ester
40.86	Octadecenoic acid, methyl ester
47.60	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester
51.68	2,6,10,14,18,22-Tetracosahexane,2,6,10,15,19,23-hexamethyl-(all-E)-
53.07	Hexadecanoic acid, 1a,2,5,5a,6,9,10,10a, octahydro-E-5a-dihydroxy-4-hydroxymethyl)-1,1,7,9-tetramethyl-
55.58	Cholesta-5,22-dien-3-ol, (3 β)-
56.30	Cholesterol
58.33	Cholest-5-en-3-ol,24-propylidene, (3 β)
60.63	17 β -Acetoxy-1',1'-dicarboethoxy-1 β ,2 β -dihydrocycloprop [1,2] -5 α -androst-1-en-3-one

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