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CHARACTERIZATION OF AN ANTIMICROBIAL AND ANTIOXIDANT COMPOUND FROM A MARINE BACTERIUM GSA10 ASSOCIATED WITH THE SPONGE *HALICHONDRIA GLABRATA*

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ABSTRACT

Three marine sponges- *Halichondria glabrata, Sigmadocia petrosioides* and *Pseudosuberitus andrewsi* were collected for isolation of sponge-associated bacteria from Mumbai coastal regions. Among 110 isolates, six strains were selected and studied for its antibacterial activity by disc-diffusion assay. TLC- Bioautography studies were performed. GSA10 isolate from *Halichondria glabrata* was selected for showing good antagonistic activity for *E. coli* MTCC-1687, *P. aeruginosa* MTCC- 1688, *B. subtilis* MTCC- 441 and *S. aureus* MTCC - 737. Various biochemical tests and 16S ribosomal RNA sequencing were performed for GSA10 for its identification which confirmed it to be a *Bacillus* species. To decipher the activity of GSA10 bacteria, we aligned the 16S rRNA sequence in NCBI database. It represented close relationship with pG1 *Bacillus amyloliquefaciens* and hypothetical protein ATP synthase from *Staphylococcus aureus*. TLC direct bioautography was developed and results were found positive for a specific band at Rf 6.8. This bioactive compound was isolated by preparative TLC and characterized by UV, FT-IR, and LC-MS. It was further evaluated for antioxidant activity. It showed good antioxidant activity in DPPH scavenging (99%) and TRAP assay (91%).The purified compound was characterized as a C-30 hopanoid from a marine-sponge associated bacteria.

Keywords: Antimicrobial activity, Bioautography, Hopanoids, LC-MS

INTRODUCTION

The marine environment is one of the most complex ecosystems with an enormous diversity of marine organisms living in close associations. Among them, eukaryotic and microorganism's association is understood to play an important role in mimicking the production of the secondary metabolites and thus these microbial associates serve as an alternative source of bioactive compounds (**Egan *et al.*, 2008**). Marine eukaryotes have been explored sufficiently for their ability to produce bioactive compounds in large-scale production, but face difficulty due to their insufficient ability to survive in mass culture. This limits their availability for future use and research (**Molinski *et al.*, 2009**). As an alternative, symbionts and marine eukaryotes associated microorganisms can be easily cultured and grown in fermenters and therefore represent the best sustainable source for biologically active compounds (**Sarkar *et al.*, 2008**). Currently, when the exact nature of the relationship between the marine host and their microorganisms remain unclear, it has been hypothesized that the microbial partners build chemical microenvironments within the eukaryotic host and lives in syntrophy, participating in cycling of nutrients. They also prevent entering of predators in the host by producing bioactive molecules forming a defensive mechanism (**Sharp *et al.*, 2005**). The close metabolic association between microorganism and their host makes it difficult to reveal the responsible mechanism for the production of a particular metabolite. Most of the marine sponge's porous body harbor dense and highly diverse microbial communities. These bacteria generally belong to phyla - Proteobacteria, Actinobacteria, Nitrospira, Chloroflexi, Planctomycetes, Cyanobacteria and Acidobacteria as well as both major lineages of Archaea and a range of unicellular eukaryotes such as diatoms and dinoflagellates. These microbes are often specific to sponges, with many microbial phylotypes appearing to live exclusively within sponge hosts and not in the surrounding seawater (**Schmitt *et al.*, 2008**). These microorganisms exhibit diverse metabolic traits useful to the host such as nitrification, photosynthesis, anaerobic metabolism and secondary metabolite production. The bioactive compounds from sponges in few cases, after investigation have shown to be produced by the microbes, rather than the sponge itself. In one of the studies, **Zheng *et al*. (2005)** isolated twenty-nine marine bacterial strains from the sponge *Hymeniacidon perleve* among which eight strains inhibited the growth of terrestrial microorganisms. Among them, the strain NJ6-3-1 was identified as *Pseudoalteromonas piscicida* with wide antimicrobial spectrum and the major antimicrobial metabolite norharman was identified (a beta-carboline alkaloid) by EI-MS and NMR. The role of these diverse microbes in sponge biology varies from source of nutrition to mutualistic symbiosis with the sponge (**Kennedy *et al.*, 2009**). A developing sponge acquires bacterial symbionts mainly by two pathways, firstly by selective absorption of specific bacteria from the surrounding water that passes through and the second one is by vertical transmission of symbionts through the gametes of the sponge by inclusion of the bacteria in the oocytes or larvae (**Radjasa *et al.*, 2007**).These pathways allow diverse microbial community to reside inside marine sponges due to its porous body. Several researchers have attempted to culture microorganisms from invertebrates with hope of obtaining bioactive invertebrate metabolites. These attempts have been successful in discovering novel natural products with rare demonstration of the presence of sponge metabolites in the microbial isolates. The suspicion about the origin of the cytotoxic compounds discovered in sponges were also established to be produced by their associated microorganisms which resulted into many other associated microorganisms to be the bioactive compounds producer (**Konig *et al.*, 2006**; **Penesyan *et al.*, 2010**). Different polyketides and nonribosomal proteins (that are generally associated with bacterial metabolism) with potent anti-cancer properties, such as halichondrin B, bryostatin and discodermolide have been also isolated from associated microorganisms of marine sponges. Specifically for bryostatin, the microbial origin was demonstrated by the identification of polyketide synthase genes involved in its biosynthesis in the genome of the bryozoan bacterial symbiont *Candidatus endobugula sertula* (**Sudek *et al.*, 2007**). In another study, a peptide antibiotic andrimid was found in a *Hyatella* sp. sponge as in a *Vibrio* sp. cultured from that sponge (**Haygood *et al.*, 1999**). *Halichondria okadai* and *Halichondria melanodocia* species of *Halichondria* contain the protein phosphatase inhibitor okadaic acid. It was first isolated from the sponge *Halichondria okadai*, but later was found out to be produced by dinoflagellate *Prorocentrum lima* (**Kelecom, 2002**). Two unidentified bacteria of the genera *Pseudomonas* and *Alteromonas* isolated from *Halichondria okadai* homogenates i.e. *Pseudomonas* sp. KK10206C also produced a novel C50-carotenoid, okadaxanthine and *Alteromonas* sp. was responsible for the production of a well-known lactam Alteramide A (**Shigemori *et al.*, 1992**, **Bhalla *et al.*, 2002**, **Thomas *et al.*, 2010**).

With the aim of finding new bioactive compounds from marine sponge associated microorganisms, we investigated *Halichondria glabrata* associated

microorganism and their compounds for antimicrobial activity were further characterized for its structure. In this study we also searched for gene which is responsible for new antimicrobial compounds from sponge-associated microbes by using BLAST and FASTA search of sponge *Halichondria glabrata.* Herein, we report comprehensive overview of protein responsible for antimicrobial compound from sponge *Halichondria glabrata* and its associated bacteria based on 16S rRNA sequencing. We aligned 200 nucleotide sequences of 16S rRNA gene with NCBI database.

MATERIALS AND METHODS

Collection of sponge material and its identification

Three different marine sponge samples were collected from intertidal zone of Khar Danda (19°4'37"N 72°49'25"E) rocky beach in western coastal region of Mumbai in the month of April, 2014 (at 16.36 hr. IST/ low tide 0.36m). Five samples of each genera were collected from the site. Sponges were removed from the substratum with a knife using protective gloves. Samples were well rinsed three times with sterile seawater in order to remove non-attached bacteria. Samples were immediately preserved in frozen condition at -80°C till further use. Samples were sent for identification to Zoological Survey of India, Chennai and are registered in Marine Biology Regional Centre, ZSI. Fragments of sponge were placed in flasks, directly on glass slides. Several drops of acid were placed on the fragment, gently heated over a flame until bubbling, and repeated until all organic matter was digested. Once it was dry and cool, preparations were mounted immediately for identification without washing (**Hooper, 2003**).

Isolation and maintenance of microorganisms

One gram of each sponge, freshly collected was weighed and triturated in mortar with pestle using sterile sea water in aseptic conditions. From the stock solution, further dilutions of 10^{-3} and 10^{-5} were made in sterile sea water. Later, each dilution of three sponges were spread plated and incubated for 20 days at 25°C.Isolated colonies were grown and preserved in marine agar (**Zheng *et al.*, 2005**).

Microbial culture preparation

Each colony from the petri plates were further grown separately in test tubes containing marine broth at 200rpm and25°C for 24 hours. The grown culture of bacteria was filled in sterile eppendorf tubes and centrifuged at 10,000 rpm for 10 minutes.

Invitro antibacterial activity of microbial extracts

The aqueous supernatants of microbial cultures were extracted in ethyl acetate solvent for the antibacterial activity of secreted bioactives by disk diffusion assay against *E.coli* (MTCC-1687), *Pseudomonas aeruginosa* (MTCC-1688), *Bacillus subtilis* (MTCC-441), *S.aureus* (MTCC-737) on marine agar. To characterize the produced antimicrobial compound, the supernatant was washed by ethyl acetate and vacuum dried. The sterile disks were loaded with 10µL of extract from each microbial culture. Ethyl acetate was used as control. The plates were kept aside for 10 hours at 8°C for diffusion process to take place. Later the plates were incubated at 37°C for 24 hours. The zone of inhibition around the disks were measured (**Jaganathan *et al.*, 2013**).

Identification of bacteria by biochemical methods and 16S rRNA sequencing

Different biochemical tests were performed for the identification of bacteria which showed positive antimicrobial activity in the disk diffusion assay (**Mukherjee, 1998**). The characterization was based on various staining and selective/differential media like gram staining, methyl red, indole test, Voges-Proskauer, catalase test, citrate test, urea test, eosin-methylene blue agar (EMB) and MaConkey agar, cetrimide agar, *Salmonella shigella* agar, triple sugar iron test and motility test. The bacteria with antimicrobial spectrum was identified to the species level by 16S rRNA gene, BLAST search and comparison with the sequences in the GenBank nucleotide sequence. The 16S rRNA gene was specifically amplified using primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'- TACGGYTACCTTGTTACGACTT-3'). The PCR conditions were kept same as described by Pandey et al., 2014.16S rRNA sequences were analysed using primers 27f and 1492r at geneOmbio Technology Pvt Ltd. Using BLAST in the GenBank nucleotide sequence. 16S rRNA sequence were analyzed by using BLAST Tree View and neighbour joining tree method at 0.85 maximum sequence difference on NCBI database having Query ID lcl|Query_178799. Alignments and phylogenic analysis were performed using multiple sequence alignment tool and ClustalX and TreeViewX software (**Larkin *et al.*, 2007**; **Tamura *et al.*, 2011**).

Genomic DNA isolation from GSA10

A single colony of GSA10 associated with the sponge *Halichondria glabrata* was inoculated into 10 ml of the nutrient broth medium and was grown by incubating overnight at the temperature of 37°C. Later, the culture was harvested by centrifugation at 5000 rpm for 8 mins and the genomic DNA was isolated by a modified DNA isolation protocol (**Sambrook and Russell, 2001**).

Fermentative process optimization and extraction for GSA10

A fresh grown 1 ml culture of GSA10of sponge *Halichondria glabrata* was added to 20ml of fermentation media and grown for a period of about six days at 200 rpm with peptone (8g/l), glucose (3g/l), yeast extract (1 g/l), $FeSO_4$ (0.1g/l) in marine water. It was grown for 6 days at 37°C, 200 rpm with 5% inoculum. Later, it was centrifuged and the supernatant was extracted successively for three times with equal volumes of ethyl acetate. Then, the dried product was obtained by vacuum evaporation and the yield was calculated (**Anand *et al.*, 2006**).

Direct Thin layer Chromatography Bioautography for GSA10 compound

Various mobile phases consisting of solvents like dichloromethane, ethyl acetate, methanol, chloroform were tried and the bands were observed at long (365nm) and short UV (254nm).The developed plates were sterilized in UV for 15mins and then were dipped in a bacterial culture of organisms *(E.coli, P.aeruginosa, S.aureus, B.subtilis)* against which the anti-microbial activity of test compound (GSA10)was tested. The dipped plates were placed on moistened cotton bed in the petri plates and then incubated for 17hours. Later the plates were sprayed with 2, 3, 3-triphenyl tetrazolium chloride (2mg/ml) and continued incubation at 37 °C for 3-4 hours (**Choma & Grzelak 2010**; **Mcgaw *et al.*, 2013**).

Isolation and characterization of GSA10 antimicrobial compound by UV, FTIR

400µl of ethyl acetate extract was spiked on TLC plate by HPTLC injector. The compound showing the antimicrobial activity was scraped and the band was dissolved in 1.5ml of ethyl acetate. The undissolved silica was separated by centrifugation at 8000rpm at 4°C for 10 minutes. Ethyl acetate was used as blank, and the compound was scanned across the wavelength of 200 – 800nm for determining λ_{max} for GSA10. For liquid samples sodium chloride cells were used for the IR interpretation.

Antioxidant assays of GSA10 compound

Scavenging activity of the nitric oxide (NO) radical

The reaction of the GSA10 compound (20 - 100 µg/mL) with nitric oxide was checked by the nitrite detection method. The reaction mixture contained 10 mM sodium nitroprusside in phosphate buffer (pH 7.4) and the GSA10 compound was incubated at 37°C for 1 h. An aliquot was added with Griess reagent and homogenized (0.1% naphthylethylenediamine in water and 1% sulfanilic acid in 5% H_3PO_4). The concentration of nitrite was assayed by taking the absorbance at 540 nm. The absorbance was also taken for standard nitrite solutions for reference. The percentage inhibition results were expressed with reference to the non-treated control (**Konrath *et al.*, 2012**).

DPPH radical scavenging activity

The discoloration of a methanolic solution of 1, 1-diphenyl-2- picrylhydrazyl radical (DPPH), was assessed by the method reported by **Mensor *et al.*, (2001)**. The GSA10 compound was assayed in a range of 20–100 µg/mL prepared in methanol. The free radicals scavenging by the compound was evaluated spectrophotometrically at 517 nm against the absorbance of the DPPH radical. A 0.1 mM DPPH solution was freshly prepared in methanol, which served as a control. The percentage of DPPH discoloration was calculated as described earlier.

Lipid peroxidation assay

Lipoperoxidation was evaluated by thiobarbituric acid reactive substances (TBARS) tests by acid-heating reaction. The protocol was followed as described by **Draper and Hadley (1990)**. Aliquots of samples were incubated with 20% trichloroacetic acid and 0.67% thiobarbituric acid. The mixture was heated (25 min) in boiling water and then centrifuged at 3000 rpm for 15 mins. TBARS was determined by reading the absorbance of the pink-colored complex formed in a spectrophotometer at 532 nm. 1, 1, 3, 3-Tetramethoxypropane, was used as standard. All experiments were performed in triplicates. A comparative analysis of means was performed using the analysis of variance (ANOVA) and Tukey's multiple comparison test ($p< 0.05$).

Reverse Phase - HPLC analysis and LC-MS

High performance liquid chromatography was carried out using C18 column- 250 x 4.6 mm stainless steel column. Various mobile phase ratio were used for proper elution and separation of peaks. Elution peaks were detected by UV detector at wavelength of 258 nm. Injection volume was set at 20µl. Flow rate was adjusted to 1 ml/min. The sample was analysed using a Perkin Elmer HPLC system equipped with a UV detector with a 200 LC pump, a 200 UV-Vis photodioarray

detector and a 200 auto sampler. The HPLC system was equipped with a degasser and an auto injector and data was collected and analysed using Totalchrom navigator software. The dried compound from GSA10 was dissolved in HPLC Grade Methanol and was run in isocratic solvent system (85%methanol, 15% water) at a concentration of 1 mg/ml after filtration with 0.45 µm Ultipor N66 Nylon 6, 6 membrane filter.

LC-MS analysis was carried out with an ion trap mass spectrometer (TDM labs, Mumbai) in both positive and negative electron spray ionization (ESI) source system. The interface was adjusted to the conditions, ion mode- positive & negative, spray voltage- 3.5 KV, capillary temperature- 325°C and auxiliary gas (nitrogen) at40 psi referring to earlier method (**Sessions *et al.*, 2013**).

RESULTS

Marine sponge

The collected marine sponge samples were identified as *Halichondria glabrata* (Dendy), *Sigmadocia petrosioides* (Dendy) and *Pseudosuberitus andrewsi* (Kirkpatrick) at Zoological Survey of India (ZSI), Chennai by acid digestion method (**Pallela *et al.*, 2011**).All the three specimen of marine sponges belonged to three different families i.e. Halichondriidae, Chalinidae and Suberitidae resp. The voucher specimen are maintained in Marine Biology Research Centre, ZSI with registration no. S-167a, S-165 and S-166 resp.

Isolation of sponge associated bacteria and their antibacterial activity

Spread plate technique was used to get isolated bacterial colonies from all the three sponge genera. Morphological characteristics of the bacterial colonies were recorded. In this study, a total of 110 marine bacteria from three sponges genera were isolated from plated depending on their distinct morphology and colony colour and shape. All the colonies were restreaked and resulted into further isolates. 73 isolates from *Halichondria glabrata*, 17 from *Sigmadocia petrosioides* and 20 from *Pseudosuberitus andrewsi* were isolated and are preserved as glycerol stock at -80°C. When screened for antimicrobial activity, few isolates from *Halichondria glabrata* and *Cliona lobata* showed positive activity represented by a clear zone around the colonies on the test microorganisms. Four isolates from *Halichondria glabrata* and two from *Cliona lobata* exhibited antimicrobial properties and were selected for characterization. The standard disks of Streptomycin, penicillin and erythromycin were used as positive control. The zone of inhibition is shown for the crude supernatants extracts of microorganisms isolated from both the sponges *Halichondria glabrata* and *Cliona lobata* (Table 1). Among all the isolated strains from sponges, GSA10 from *H. glabrata* showed good antibacterial activity and thus were evaluated for its bioactive compound.

Table 1 Zone of inhibition of strains showing activity

Sr.no.	Name of marine sponge	Test organism	*E.coli*	*P. aeruginosa*	*B. subtilis*	*S. aureus*
		Strains isolated	MTCC- 1687 (mm)	MTCC- 1688 (mm)	MTCC- 441 (mm)	MTCC- 737 (mm)
1		GSA10	8.67±0.47	8.33±0.47	5.5±0.41	6.23±0.21
2	*Halichondria glabrata*	GS1C	8.83±0.62	-	-	-
3		GSB10	4±0.00	-	-	-
4		GS2	10.16±0.15	-	-	-
5		BS 2	-	10.5±0.41	-	-
6	*Cliona lobata*	BS 1	9±0.00	-	-	10.3±0.21
		Standard Antibiotic				
		Penicillin	10.53±0.36	-	10.07±0.09	40.47±0.37

˟ indicates no or minimal activity, results are Mean+ SEM.

Biochemical characterization of isolated microbial strains

In our study, the strains with bioactivity showing zone of inhibition were gram-negative rods (GSB10, GS2 & BS2) and gram-negative cocco-bacilli (GSA10, GS1C & BS1). GSA10 and GS1C colonies were round in shape and white (opaque) in colour, whereas GSB10 and GS2 were rhizoid shaped and white in appearance. BS2 colonies were circular, white and sticky. BS1 colonies were irregular, white and sticky in appearance. Further, biochemical tests- indole test, methyl red test, Voges-Proskauer, catalase test, citrate test, urease test, triple sugar iron test , motility test and selective medias like eosin-methylene blue, MacConkey's and Cetrimide agar were also performed to characterize the isolated bacteria. Biochemical tests confirmed the predominance of Gram-negative associates in the marine sponges (Table 2).According to the results of biochemical tests, the strain GSA10 either belonged to *Enterobacteriaceae* or *Proteus* species (**Patel & Patel 2004**, **Mukherjee 1988**, **ABIS online software- www.tgw1916.net**). ABIS online software identified GSA10 with % probabilities as *Proteus penneri* ~ 87%, *Morganella moraginii* ~ 87%, Enteric group 60 ~ 87% and *Enterobacter pyrinus* ~ 86%. For genomic confirmation, GSA10 was subjected to 16S ribosomal RNA sequencing upto 770 bp. The assembled FASTA format sequence confirmed GSA10 to be a *Bacillus* strain with 100% similarity generated by NCBI BLAST search with accession no. GenBank KT750840. The sequence was later classified using RDP Naive Bayesian rRNA Classifier Version 2.10 for taxonomical hierarchy. The classifier_seq confirmed the GSA10 to be a *Bacillus* with +; Root 100%; Bacteria 100%; Firmicutes 100%; Bacilli 100%; Bacillales 100%; Bacillaceae 1 100%; *Bacillus* 100% (**Wang *et al.*, 2007**).

Table 2 Results of biochemical test performed on the isolated strains

S.No.	Biochemical Test	GSA10	GSB10	GS1C	GS2	BS2	BS1
			Halichondria glabrata				*C. lobata*
1.	Indole test	-ve	-ve	-ve	-ve	-ve	-ve
2.	Citrate test	-ve	+ve	-ve	+ve	+ve	-ve
3.	Methyl red test	-ve	-ve	+ve	-ve	-ve	-ve
4.	Voges- Proskauer test	-ve	-ve	-ve	-ve	-ve	-ve
5.	Catalase test	+ve	+ve	+ve	+ve	+ve	+ve
Tests carried out only for strain GSA10							
6.	Triple sugar iron test	+ve	-	-	-	-	-
7.	*Salmonella shigella* test	-ve	-	-	-	-	-
8.	Cetrimide test	-ve	-	-	-	-	-
9.	Urea test	+ve	-	-	-	-	-
10.	Motility test	+ve	-	-	-	-	-

Thin layer chromatography

The proper separation of bands for the ethyl acetate extract of strain GSA10 was optimized with the mobile phase dichloromethane, ethyl acetate, methanol (5:5:1) and UV/Vis chamber was used for detection at wavelengths of 254nm and 365nm.Total six bands with R_f at 0.27, 0.378, 0.513, 0.594, 0.68 and 0.72 were observed.

Direct TLC Bioautography

Direct TLC Bioautography method was primarily tested and optimized for *E. coli* (MTCC-1687), *P. aeruginosa* (MTCC-1688), *Bacillus subtilis* (MTCC-441) and *S. aureus* (MTCC-737) as test strains. 2-Phenyl tetrazolium salt (2mg/ml) was used as spraying agent. 20µl and 50µl of test antimicrobial compound was used for loading on TLC plate in order to see the effective concentration to kill the bacteria. Strain GSA10 antimicrobial activity was found to be positive for *E.coli, B. subtilis* & *P. aeruginosa*. Increase in the incubation time gave more homogeneous and dark pink-violet coloured sharp contrast inhibition zones showing presence of antimicrobial compound. The band at R_f = 0.68 showed good antimicrobial activity

(Fig. 1 and Fig 2). The band compound was taken for Prep-TLC run plate with 400 µl of sample.

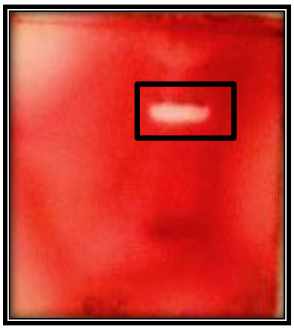

Figure 1 TLC-DB for strain GSA 10 compound (*B.subtilis*)

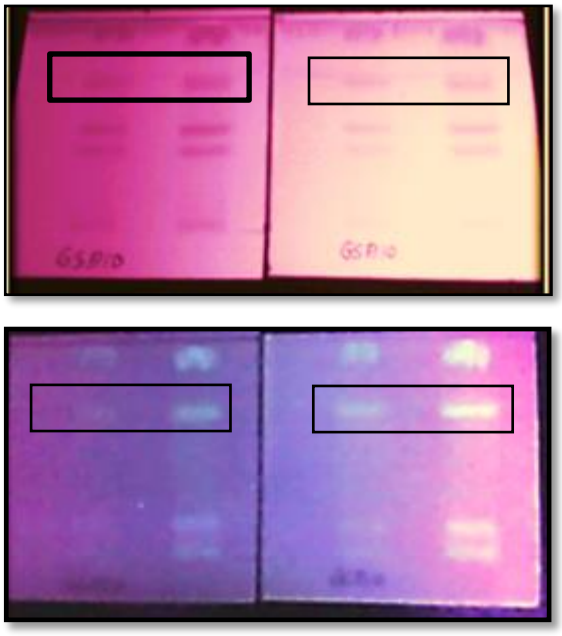

Figure 2 HPTLC of ethyl acetate extract of strain GSA10 at a) 254 nm & b) 365 nm

Antioxidant activity

Table 3 summarizes the antioxidant effects of GSA10 against nitric oxide production, DPPH discoloration and lipid peroxide antioxidant potential. At the final concentration of 100 µg/mL, the inhibitory power against nitric oxide production was found to be 52.3 + 0.09 %. The compound could also reduce the DPPH radical at all tested concentrations. It gave 99.62 + 0.16 % inhibition at 100 µg/mL. In the TRAP assay, the GSA10 acted as peroxyl scavengers, and the % inhibition were proportional to the GSA10 concentrations added. It showed maximum total reactive antioxidant potential of 91.95 + 2.74 % at the highest concentration.

Table 3 Inhibition profile of GSA10 compound against nitric oxide production, DPPH radical scavenging activity and total reactive antioxidant potential (TRAP) assays as free radical scavengers

Compound	Dose (µg/mL)	Nitric oxide production	DPPH scavenging	TRAP assay
GSA10	20	29.8 ± 0.12*	78.23 ± 0.12**	79.6 ± 3.25**
	40	37.8 ± 0.14*	82.93 ± 0.4*	82.5 ± 2.53*
	60	43.8± 0.12**	88.26 ± 0.2***	85.13 ± 2.17*
	80	45.7 ± 0.24**	98.23 ± 0.2*	89.4 ± 1.14**
	100	52.3 ± 0.09*	99.62 ± 0.16**	91.95 ± 2.74*

Data are expressed as mean ± standard deviation (n = 3); mean in the same column with different superscripts are significantly different using Duncan's multiple range test at p <0.05.

GSA10 antimicrobial compound characterization

The λ_{max} for the compound was found to be 258.94nm with A= 0.078. The IR spectra gave absorption at 2980 corresponding to $-CH_3$, signal at 1732 indicated presence of carboxylic acid derivatives (ester). Absorption at 1432 indicated aromatic (C=C) and 1360 for alkanes stretch (C-C) and 1032 was showing carboxylic acid (C-O) functional groups presence (Fig 3). TLC spot gave violet colour with freshly prepared p-anisaldehyde- sulphuric acid reagent which suspected the presence of steroids or terpenes.

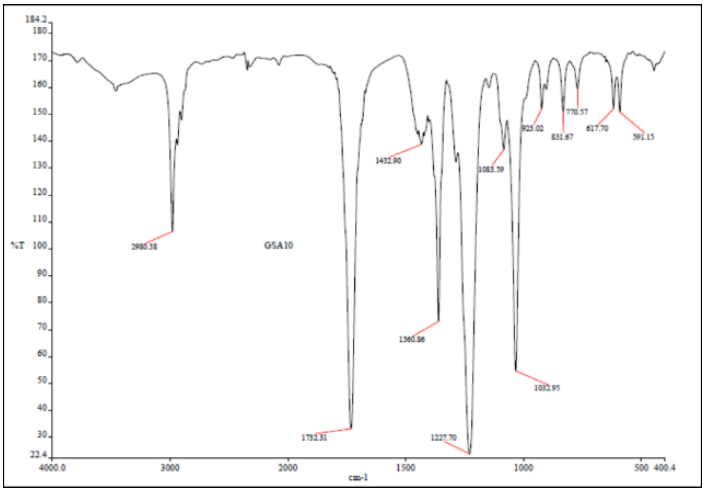

Figure 3 IR Spectra of ethyl acetate extract of strain GSA10

LC-MS Characterization

HPLC method development resulted in best separation and resolution peak with mobile phase methanol: water (85:15) at RT 11.55. LC-MS was performed for the isolated bioactive compound to characterise the structure and molecular weight. LC- MS spectra showed different fragments in positive and negative mode. $[M^+]$

was observed at *m/z* 414 in both mode, indicating it to be a molecular ion. Major ions were present at *m/z* at 366, 325.9, 312, 300, 269.5, 255, 239.6, 198.3, 186.8 and 99.4 (Fig. 4) in negative mode. Spectra peaks of solvent methanol were nullified for characterization of compound. These ions represented the fragments of a hopanoid. In Fig 5, LC-MS we found peaks at *m/z* 186.8, *m/z* 366, *m/z* 340, *m/z* 229.1 which represented fragment ions of Hop-21-ene (**Sessions *et al.*, 2013**). In another study, GC- MS of the sterol fraction has shown the characteristic *m/z* 191 and 215 ions as significant peaks of a hopanoid (**Silipo *et al.*, 2014**). The structure of bioactive compound was also justified by the fragmentation pattern of the hopanoid nucleus with probable structures of various *m/z* ions in spectra as shown in Fig.5.

BLAST study

16S rRNA sequence were analyzed by using BLAST Tree View and neighbour joining tree method at 0.85 maximum sequence difference on NCBI database having Query ID lcl Query_178799. Alignments and phylogenic analysis were performed using multiple sequence alignment tool and ClustalX and TreeViewX software (**Larkin *et al.*, 2007**; **Tamura *et al.*, 2011**) the results showed that the GSA10 culture of bacteria closely related to *Bacillus amyloliquefaciens, Bacillus pseudofirmus, Anoxybacillus* sp. UARK-01, *Anaerobacillus alkalilacustris* and *Staphylococcus aureus* subsp. *aureus MN8.* The blast names colour map is enclosed in the Fig. 6. It represents close relationship with pG1 *Bacillus amyloliquefaciens* and hypothetical protein ATP synthase from *Staphylococcus aureus.* GSA10 is rooted tree in which round grey circle (•) represents nodes and coloured triangle (Δ) represents bacterial species known as leaf. The protein sequence of GSA10 and its putative functions of the relevant proteins were deduced by *in silico* analysis using SMARTBLAST. The GSA10 species is related to five species namely *Bacillus amyloliquefaciens*, *Bacillus pseudofirmus, Anoxybacillus* sp. UARK-01, *Anaerobacillus alkalilacustris* and *Staphylococcus aureus* subsp. *aureus* MN8 (Table 4). The antibacterial compound extracted from GSA10 is 100% identical with pG1 protein [*Bacillus amyloliquefaciens*], 99% identical with hypothetical protein BTR23_20630 [*Bacillus pseudofirmus*], 94% identical with hypothetical protein B6A27_12100 [*Anoxybacillus* sp. UARK-01] and hypothetical protein BKP37_01205 [*Anaerobacillus alkalilacustris*]. The antibacterial compound extracted from GSA10 is only 91% identical with hypothetical protein HMPREF0769_11381 [*Staphylococcus aureus* subsp. *aureus* MN8].

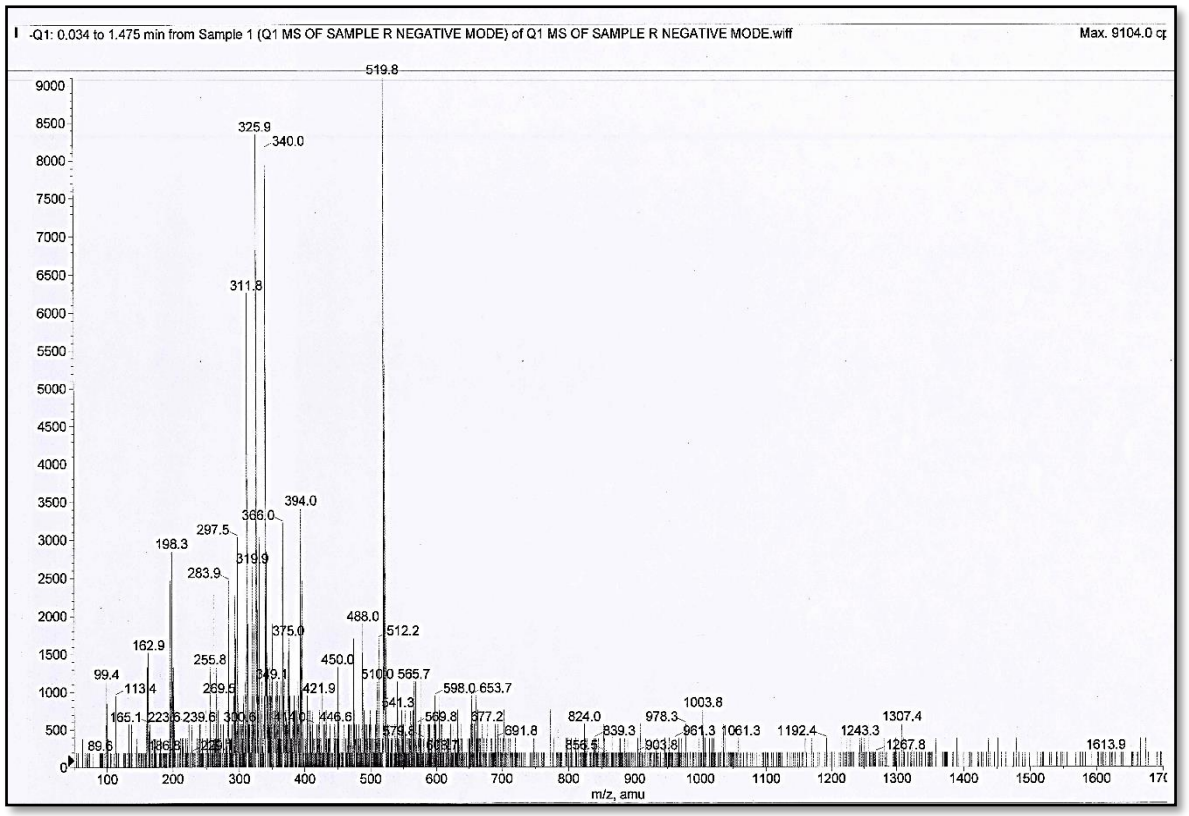

Figure 4 LC-MS spectra for the GSA10 compound

Table 4 The putative functions of relevant genes by *in silico* analysis using SMARTBLAST.

Sr. No.	Accession	Description	Expect value	Identity
1	ACX55038.1	pG1 protein [*Bacillus amyloliquefaciens*]	4e-50	100%
2	OLO27204.1	hypothetical protein BTR23_20630 [*Bacillus pseudofirmus*]	1e-49	99%
3	OQM45369.1	hypothetical protein B6A27_12100 [*Anoxybacillus* sp. UARK-01]	7e-47	94%
4	OIJ17655.1	hypothetical protein BKP37_01205 [*Anaerobacillus alkalilacustris*]	1e-43	94%
5	EFH95998.1	hypothetical protein HMPREF0769_11381 [*Staphylococcus aureus* subsp. *aureus* MN8]	6e-43	91%

DISCUSSION

H. panacea has been reported to harbour bacteria of various genera with *Rhodobacter* genus being the most dominant suggesting their and other species symbiotic relationship with the sponge host (**Althoff *et al.*, 1998**).The growth of these bacteria in *Halichondria* was understood to be maintained due to a lectin produced by the host sponge. As there has been limited success with cultivation of sponges in laboratory, the sponge-specific microorganisms can serve for the current genomics revolution (**Schippers et al., 2012**). Of the plethora of bioactive natural products found in them, the terpenoids are prominent and widespread. Several novel meroterpenoids, including a new structural group of meroterpenoid metabolites, the insuetolides A-C as well as several drimane sesquiterpenes, were recently isolated from the marine-derived fungus *Aspergillus insuetus* isolated from the Mediterranean sponge *Psammoncinia* sp. (**Orhan *et al.*, 2010**). Pentacyclic triterpenoid also known as hopanoids are widely distributed in prokaryotes but are not detected in Archaea (**Rohmer *et al.*, 1984**). They are found predominantly in aerobic methanotrophs, heterotrophs and cyanobacteria and in some anaerobic bacteria too (Sinninghe et al., 2004). Since its first finding, they have been found to be present in nature in vast amounts as bacterial and other primitive organism's components. Many bacterial groups also possess unique and recognizable biohopanoid distributions, making them marker for specific bacterial populations. A range of hopanoids found in petroleum reservoirs were also used as biological markers (Hunt 2002). In one of the study, Plakohopanoid, a new type of hopanoid composed of a C32 hopanoid acid ester linked to a mannosyl-*myo*-

inositol was isolated from the sponge *Plakortis* cf. *lita*. Even it was isolated from a sponge, its component parts clearly indicated it to be of bacterial origin, and very likely via bacterial biosynthesis (**Costantino *et al.*, 2012**).Hopanoids have been reported to control membrane fluidity and diminish passive diffusion of ions. Few taxons modulate their hopanoid content in response to environmental stimuli (**Seipke and Loria, 2009**). **Wu *et al.*, (2015)** recently reported that hopanoid 2-methylation specifically renders native bacterial membranes more rigid using fluorescence polarization studies of small unilamellar vesicles. Hopanoids differentially modified native membrane rigidity as a function of their methylation state which indicated that methylation promotes fitness under stress. Bacteriohopanetetrol also promotes order within the lipid tails in membranes but enhances fluid-like properties of the head groups at high temperatures. In contrast, diplopterol partitions in the midplane of the bilayer. Individual hopanoids fulfil distinct functions in membranes with the ordering properties of bacteriohopanetetrol particularly to maintain the integrity of membranes at temperatures preferred by thermo tolerant and thermophilic bacteria (**Caron *et al.*, 2014**).Another study indicated that hopanoid production might be common, but not obligate in the genus *Desulfovibrio*. In contrast to the other strains of sulphur reducing anaerobic bacteria, *Desulfovibrio* and *D. bastinii* contained high amounts of nonextended hopanoids and bacteriohopanepolyols; with diploptene, 17β(H),21β(H)-bacteriohopane-32,33,34,35-tetrol and 17β(H),21β(H)-35-aminobacteriohopane-32,33,34-triol being the major compounds. This moderately halophilic *D. bastinii* was isolated from a deep subsurface oil formation water (**Blumenberg *et al.*, 2009**). **Haines (2001)** speculated that hopanoids fulfil a unique role in acidophile membrane structure by preventing inward leakage of protons. **Lopez- Revuelta (2005)** and colleagues found that depletion of cholesterol from RBCs increased their vulnerability to peroxidation which reversed by cholesterol repletion. The role of sterols in free radicals protection can be understood as they are in an ideal position to encounter ROS like hydrogen peroxide (H_2O_2) that crosses this permeability barrier and indicates its connection with antioxidant property (**Murphy and Johnson, 2008**). Prokaryotic hopanoids are understood to be sterol surrogates and in our study the isolated hopanoid displayed good antioxidant property. The study showed that the hopanoid can be a good antioxidant molecule as they gave good percentage inhibition in two invitro models i.e. DPPH scavenging and TRAP assay. With such antibacterial and antioxidant potential the GSA10 compound was characterized using spectroscopic method. In the BLAST study we correlated pG1 partial protein of *Bacillus amyloliquefaciens* and ATP synthase subunit alpha domain protein from *Staphylococcus aureus*. pG1 is protein coding gene known as polygalacturonase 1. pG1 protein is located in endomembrane system of chromosome. This enzyme plays important role in homogalacturonan degradation, pentose-glucuronate interconversions, metabolic pathways, and in plant defence. They act by releasing elicitor and acts as cell wall degrading enzymes (**Mariotti *et al.*, 2009**). This may be one reason for GSA10 showing good antimicrobial activity. On other side pG1 also shows close relationship with ATP synthase subunit alpha domain protein *Staphylococcus aureus*.

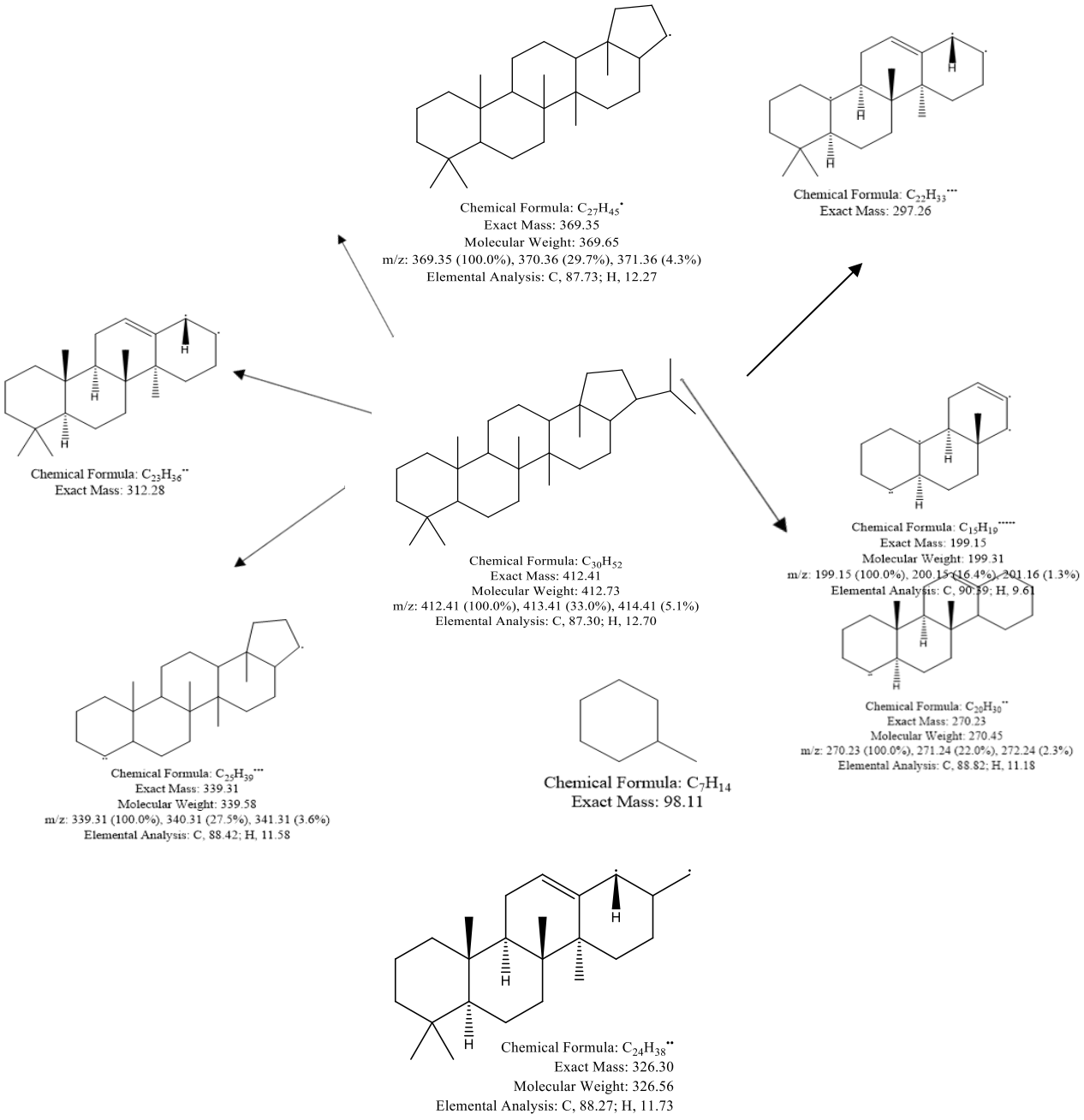

Figure 5 Predictive fragment ions of different m/z of GSA10 bioactive haponoid compound shown in mass spectra.

APGC is a 'soft' ionization technique which results in lower fragmentation, whereas electron impact (EI) mode gave extensive fragmentation resulting in high abundance of low mass fragments. A typical APGC-MS fragments observed for hopanes were *m/z* 177, 191, 369 and C_{30} hopane has the precursor ion (*m/z* 412/413/414) (**Hancock *et al.*, 2013**; **Sessions *et al.*, 2013**). GSA10 hopanoid also gave hopane characteristic fragments at 367 and 414 in negative ionization, which confirmed it to be a C_{30} hopane. GSA10 a sponge associated bacteria which was characterized and identified as a *Bacillus* species, showed antibacterial activity. The secondary metabolite was found to be a C30 hopanes and suggests investigating its mode of action for showing this inhibition against terrestrial microorganism. There are few reports found on hopanes antibacterial activity.30-Ethyl-2α, 16α-dihydroxy-3β-O-(β-D glucopyranosyl)-hopan-24-oicacid from plant and

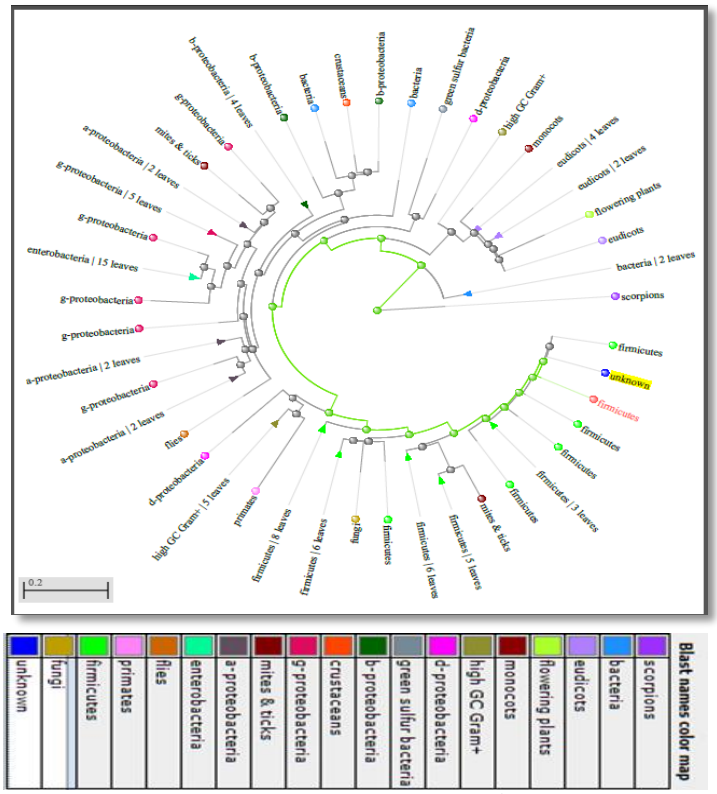

Figure 6 Neighbour joining circular dendrogram of GSA10 antibacterial compound

Hopan-27-al-6β, 11R, 22-triol from fungi have already shown *E.coli* and *P. aeruginosa* and *Mycobacterium tuberculosis* (**Semwal *et al.*, Isaka *et al.*, 2011**).These observations suggests that ATP synthase may supply energy during the cell was degradation. The pG1 and ATP synthase both are interlinked as given in phylogenic tree. GSA10 compound is closely related to these species. Hence we can conclude that the ATP synthase provide energy during cell wall degradation and due to which GSA10 shows antibacterial activity (**Preuss *et al.*, 2011**) (**Bernardi *et al.*, 2015**). For analysis of hopanes and steranes, a study was performed by Hancock et al., 2013 to analyse petroleum biomarkers by ionization using Waters Atmospheric Pressure Gas Chromatography (APGC-MS/MS). Further studies can be planned to elucidate the conformational structure using 2D NMR. At the same time, determination of minimum inhibitory concentration against the test organisms will also help to assess the future scope of these secondary metabolites in antibacterial therapy

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