Volume 65, Issue 5, 2021
Journal of Scientific Research
of
The Banaras Hindu University

Screening And Bioactivity Profiling of Marine *Bacillus* Species Against Human Pathogens

J. Thelma[1]*, Chellaiah Balasubramanian[1]
[1*] Department of Zoology, Thiagarajar College, Madurai, Tamil Nadu, India
[1]Department of Zoology, Thiagarajar College, Madurai, Tamil Nadu, India
[1]*Present Address: Department of Zoology, Fatima College, Madurai, Tamil Nadu, India
E-mail: thelmaphd2016@gmail.com*

Abstract: **Screening and characterization of the secondary metabolites from the marine microbial organisms helps in the identification of potential bioactive molecules with pharmaceutical importance. Hence, in the present study, a total of seven marine samples were collected from different sampling sites around Tamilnadu and Andaman, out of which 39 isolates were screened for antagonistic against five human pathogens. Among the isolates, two potent *Bacillus* strains (C5 & M6) exhibited antagonistic activity against pathogenic microorganisms. The maximum zone of inhibition was exhibited by C5 against *Staphylococcus aureus* (14.03±0.02mm), followed by *Bacillus cereus* (13.3±0.02mm), *Pseudomonas aeuroginosa* (11±0.08mm), *Escherichia coli* (9.03±0.01mm) and *Streptococcus mutans* (6.93±0.08mm). The strain M6 exhibited maximum zone of inhibition against *Pseudomonas aeuroginosa* (11.8±0.15mm) followed by *Staphylococcus aureus* (6.03±0.05mm), *Bacillus cereus* (5.03±0.05mm), *Escherichia coli* (4.9±0.01mm) and *Streptococcus mutans* (4.86±0.15mm). These organisms were identified as *Bacillus marisflavi* (C5) and *Bacillus flexus* (M6) by 16s rRNA gene sequencing. Comparatively, the strain C5 was more effective than M6. The bioactive components from culture broth were separated by Thin Layer Chromatography and the chemical constituents of the fractions were analyzed by Gas chromatography–mass spectrometry. This revealed the presence of numerous bioactive compounds like Phenol, 2,5-bis(1,1-dimethylethyl), Cyclododecane, Hexadecanoic acid ethyl ester, Pentadecanoic acid ethyl ester, Octadecanoic acid ethyl ester from C5 and M6. The findings suggested that marine water samples have the potent source of bioactive compounds as a novel source for drug discovery. Further, structural prediction and characterization of the bioactive compounds may lead to the discovery of novel compounds for potential medicinal applications.**

Keywords: **Marine ecosystem, Tamilnadu, *Bacillus marisflavi, Bacillus flexus*, Antagonistic activity.**

I. INTRODUCTION

A majority of the biosphere is occupied by the marine environment that contributes to 97% of the earth. The marine environment harbours a huge reservoir of marine microorganisms that have potent bioactive compounds to exploit as natural products for the treatment of human diseases (Chellaram *et al.,* 2004). The marine environment is poorly explored in terms of potential pharmaceuticals. A good source of novel metabolites can be obtained from these microbes that are tremendous in the marine ecosystem. Screening of bioactive compounds can be achieved through the study of novel microorganisms of marine origin (Sagar *et al.,* 2015). The genus *Bacillus* is a group of gram-positive rod-shaped bacteria that are heterogeneous both phylogenetically and morphologically. Extensive studies have been done in the genus *Bacillus* and are classified into four major groups (Ash *et al.,* 1991). *Bacillus* strains can survive in adverse conditions (Claus, 1986). Priest (1993) has revealed that *Bacillus* sp. are widely distributed in aquatic environments that are capable of forming spores with ability to tolerate a variety of unfavourable environmental conditions. To date, approximately 500 species belonging to the genus *Bacillus* have been reported. However, the poorly studied marine *Bacillus*, on the other hand, is even more varied (Liu *et al.,* 2017). The search for biological antimicrobials with improved bioavailability and improved efficiency, with low resistance induction, is more appropriate and appreciated

DOI: 10.37398/JSR.2021.650510

currently. Hence the present study, aims to screen marine *Bacillus* species with an antagonistic effect against human pathogens. Therefore an attempt was made to isolate and identify *Bacillus* from the marine ecosystem in and around Tamilnadu and to evaluate its antagonistic activity against human pathogenic microorganisms.

II. MATERIALS AND METHODS

A. Collection of marine water samples

Bacillus species used in this study were isolated from marine water samples collected from coastal areas from seven different sites in and around Tamil Nadu as listed in table 1. Surface water samples from a depth of about 0.3m were collected in sterile screw-cap containers and stored in the refrigerator at 4°C for further work.

Table 1: Sample collection sites in and around Tamil Nadu

S.No	Name of the site	Coastal region	Latitude	Longitude
1	Velankanni	Coramandel coast of Bay of Bengal	10.6811° N	79.8536° E
2	Mandapam shoreline	Gulf of Mannar, confluence of Indian Ocean and the Bay of Bengal	9°17'18.60" N	79°18'45.76" E
3	Marina beach	Coramandel coast of Bay of Bengal	13.0500° N	80.2824° E
4	Kanyakumari shoreline	Confluence of Arabian Sea, Indian Ocean, and the Bay of Bengal	8.0866° N	77.5544° E
5	Tuticorin	Gulf of Mannar	8.7644° N	78.1507° E
6	Andaman	Northeastern Indian Ocean	11° 37' 24.15" N	92° 43' 35.34" E
7	Tiruchendoor	Coromandel Coast off the Bay of Bengal	8.4863° N	78.1221° E4

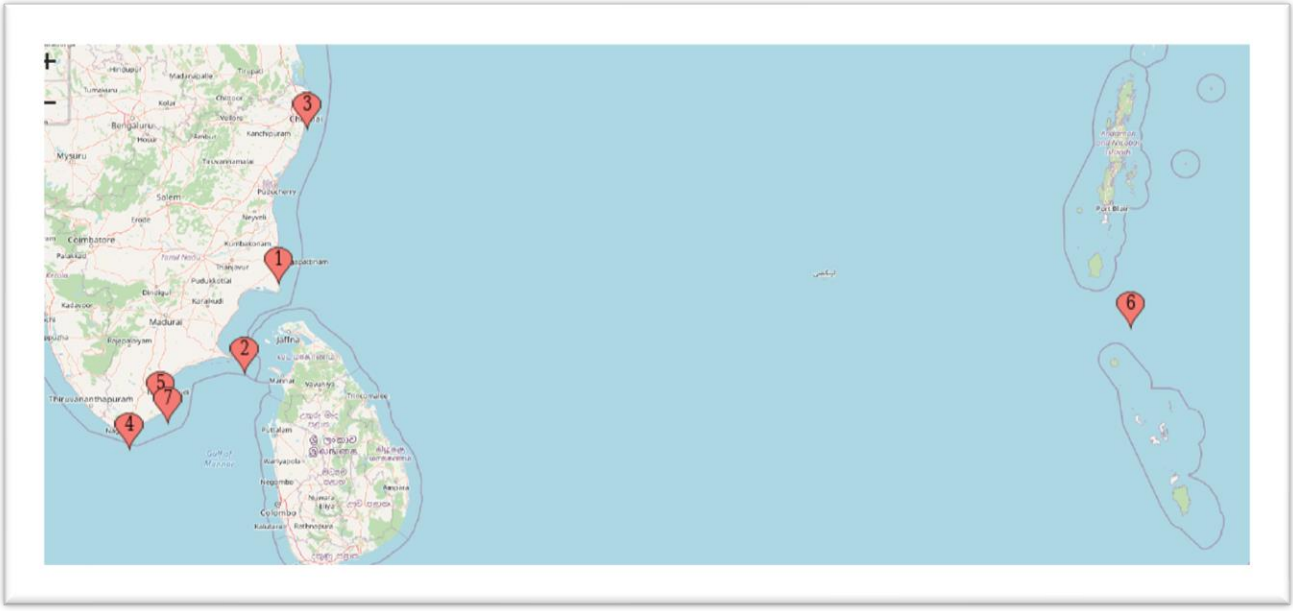

Figure 1: Map showing marine water sample collection sites in and around Tamilnadu

B. Isolation of marine Bacilli

One ml of seawater sample was suspended in 9 ml of sterile distilled water and serially diluted. For isolating the pure culture of *Bacillus*, the dilutions were incubated at 30°C for 4 hours and heat-shocked at 80°C for 3 to 5 minutes in a water bath to destroy all vegetative microbial cells. 100µl of sample from each dilution was taken and spread on Zobell marine agar (HiMedia, India). The plates were inverted and incubated at 30°C for 24 hrs. Pure colonies were identified based on morphological features. Pure colonies were picked up from the agar plate by using an inoculation loop and were smeared onto a glass slide for Gram's staining (Cappuccino & Sherman, 2002).

C. Species identification

To characterize the organism at the species level, the strains were subjected to genetic characterization by 16S rRNA gene sequencing. The Chromosomal DNA was isolated by using standard protocols. The isolated DNA was amplified by PCR with specific primers for the conserved regions of 16S rRNA following which the DNA was sequenced. The sequences were compared with sequences deposited in the 16S rRNA database. Phylogenetic analysis was done using MEGA 7 software and the phylogenetic tree was constructed using the sequences generated from this study along with 18 reference sequences retrieved from GenBank, NCBI. Tree construction was done based on the Maximum Likelihood method (ML) with a bootstrap value of 1000 times replication and gaps were considered as missing data (Tamura *et al.*, 2013).

D. Detection of antagonistic activities

The Gram-positive pathogenic bacteria *Streptococcus* mutants (MTCC –890), *Staphylococcus aureus* (NCIM–2079) and *Bacillus cereus*(MTCC–430), and the Gram-negative strains *Escherichia coli* (MTCC–46) and *Pseudomonas aeruginosa* (NCIM – 2200) were procured from Microbial Type Culture Collections (MTCC), IMTECH, Chandigarh, India and National Collection of Industrial Microorganisms (NCIM), Pune, Maharastra, India. The cultures were kept on slants and stored at 4ºC. These strains were sub-cultured every week in order to maintain the virulence. The antagonistic properties of isolated bacterial species were determined by agar well diffusion method. Mueller Hinton Agar Medium (1 L) was prepared by dissolving 33.9 g of the commercially available Mueller Hinton Agar Medium (HiMedia, India) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm Petri plates (Borosil®) (25-30ml/plate) while still molten. Petri plates containing 20ml Mueller Hinton medium (HiMedia, India) were seeded with 24hrs culture of pathogens. Wells were cut and the *Bacillus* cultures were added at varying concentrations from 100µl to 500µl. The plates were then incubated at 37°C for 24 hours. The antagonistic activity was evaluated by measuring the diameter of the inhibition zone formed around the well. The assay was done in triplicates for each isolate and the results were expressed as a mean value of inhibition zone diameter.

E. Isolation and identification of metabolites

The supernatant of the cultures that showed maximum zone of inhibition were analyzed by thin-layer chromatography (TLC). TLC plates (60G F_{254}) with precoated silica gel were used for the separation of compounds. The developing solvent was dichloromethane: ethyl acetate: methanol (5:5:1). The unknown compounds on TLC were collected and dissolved in the solvent used for elution. These partially purified compounds were analyzed again by GC and MS.

F. GC-MS analysis

The fractions eluted from TLC, were subjected to GC-MS to identify the bioactive compounds. The sample was analyzed in GC Clarus 500 Perkin Elmer by using software Turbomass 5.2 equipped with mass detector Turbo mass gold Perkin Elmer. 2µl sample was introduced via an all-glass injector working in the split mode, with Helium as the carrier gas with a linear velocity of 32 cm/s. The HP-5 fused silica capillary column (Length – 30 m; Film thickness- 25 µm I.D-0.2 mm) was used. Data assessment was performed through Turbomass software and a library search was carried out using NIST library.

III. RESULTS AND DISCUSSION

The Indian marine ecology boasts a thriving microbial diversity. In terms of prospective medications, the marine environment has received insufficient attention. It is home to an astounding diversity of organisms, making it an excellent source of new metabolites that might be employed in the treatment of infectious diseases. In the present study, a total of 7 marine water samples were collected from various marine ecosystems in

and around Tamilnadu as listed in table 1. After the serial dilution technique, only the *Bacillus* species were isolated by specific procedures. About 39 different *Bacilli* colonies were selected based on morphological variations of the colony and confirmed by Gram's staining. Strain codes were given to each colony until species identification was done (Table 2).

Table 2. Number of colonies selected from each sample site

S.No	Name of the site	No of colonies selected	Strain code
1	Velankanni	6	V1, V2, V3, V4, V5, V6
2	Mandapam shoreline	8	M1, M2, M3,M4,M5,M6,M7,M8
3	Marina beach	8	C1, C2, C3, C4, C5, C6, C8
4	Kanyakumari shoreline	3	K1,K2,K3
5	Tuticorin	4	Tu1,Tu2,Tu3,Tu4
6	Andaman & Nicobar island	8	A1, A2, A3,A4, A5, A6, A7, A8
7	Tiruchendoor	2	T1,T2,T3,T4

Fig 2: Gram's stain of C5 & M6

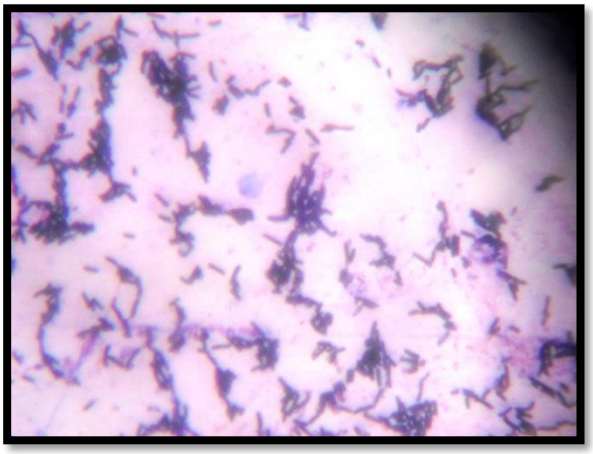

Fig 2.1 Gram stain of C5 showing Gram-positive rods

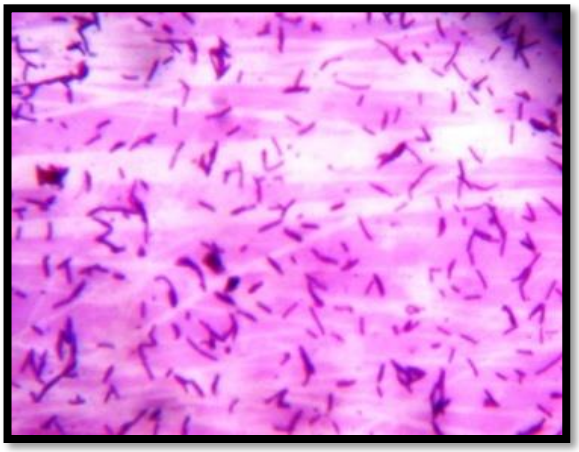

Fig 2.2 Gram stain of M6 showing Gram- positive rods

Bacillus species found in a wide range of environments are including water, dead insects, plants, soil, the marine environment, animal guts, and food samples (Krawczyk *et al.*,2015; Shewan *et al.*, 1954). The aerobic organisms are capable of producing endospores that allow them to live in the most extreme climatic conditions and they synthesize a diverse range of bioactive compounds used in the field of agricultural, fishery, medicinal, and veterinary sciences (Piewngam *et al.*,2018; Sharp *et al.*,1992; Stein, 2005). It was assumed that the extracellular secondary metabolites produced by the isolates have inhibitory activity against pathogenic microorganisms. Hence, the culture supernatant of all the 39 isolates were tested for antagonistic activity against *Streptococcus mutans* (MTCC –890), *Staphylococcus aureus* (NCIM – 2079), and *Bacillus cereus* (MTCC – 430), and the Gram-negative strains such as *E. coli* (MTCC – 46) and *P. aeruginosa* (NCIM – 2200). Finally, two potential *Bacillus* isolates with strain codes C5 and M6 were selected based on their inhibitory effect against the pathogens. Among the two potential strains, strain C5 showed the most significant activity against Gram-positive and Gram-negative pathogens. The assays were done in triplicates and the diameter of the inhibitory zones was expressed as Mean ±

Standard Error as shown in Table 4,5 and Fig 5,6. The maximum zone of inhibition was exhibited by C5 against *Staphylococcus aureus* (14.03±0.02mm), followed by *Bacillus cereus* (13.3±0.02mm), *Pseudomonas aeuroginosa* (11±0.08mm), *Escherichia coli* (9.03±0.01mm) and *Streptococcus mutans* (6.93±0.08mm) at a concentration of 0.5ml. The strain M6 exhibited maximum zone of inhibition against *Pseudomonas aeuroginosa* (11.8±0.15mm) followed by *Staphylococcus aureus* (6.03±0.05mm), *Bacillus cereus* (5.03±0.05mm), *Escherichia coli* (4.9±0.01mm) and *Streptococcus mutans* (4.86±0.15mm) at a concentration of 0.5ml. The antagonistic activity exhibited by these organisms could be due to the metabolites synthesized by the organisms extracellularly. The marine microorganisms of the Pacific Institute of Bio-organic Chemistry (Vladivostok, Russia) have taxonomically studied a group of marine *Bacillus* strains with the ability to produce biologically active compounds (Ivanova *et al.,* 1998; Jensen *et al.,* 1996). *B. subtilis* has been extensively experimented by various researchers for its anti-fungal activity (Cubeta *et al.*,1985; Ferreira *et al.*,1991; Fravel 1998; Seifert *et al.*,1987). Loeffler *et al.,* (1986) implied that the mode of antagonistic action is due to the production of antibiotics. Surfactin molecules isolated from *B. pumilis* showed inhibitory activity against *S. aureus*, *P. vulgaris*, and *E. Faecalis* (Berrue *et al.*,2009). Bioactive compounds such as 3-methylpyridazine, n-hexadecanoic acid, indazol-4-one, octadecanoic acid, and 3a-methyl-6-((4-methyl phenyl) sul Alterperylenol (Al-dhabi *et al.*,2019; Zhao *et al.,* 2018), Trypilepyrazinol (Li *et al.*,2019), Aspergillsteroid A (Xu *et al.*,2020) have been reported to exhibit antibacterial properties. Metabolites such as an antibiotic, 3-amino-3-deoxy-D-glucose (Fusetani *et al.,* 1987), a new glucanase (Okami *et al.,* 1980), and cyclic acyl peptides (Gerard *et al.,* 1996) have been isolated. Similar results were observed in the culture broth of *B. amyloliquefaciens* (FZB42) which showed the presence of Difficidin and oxidifficidin that are highly unsaturated 22-membered macrocyclic polyene lactone phosphate esters with broad-spectrum of antibacterial activity (Chen et al.,2009). *B.aerius*, *B.oryzicola*, *B.safensis*, *B.boroniphilus*, *B.altitudinis*, and *Virgibacillus senegalensis* isolated from marine environments in Mexico showed acute antagonistic action against the food-borne poisoning pathogens *S. aureus* and *Vibrio parahaemolyticus*. These marine bacteria appear to be a viable option to other clinically significant microorganisms for the development of novel antimicrobial agents (Galaviz-Silva *et al.* 2018). Dentigerumycin E, a newly found metabolite isolated from the coastline of a muddy wetland by co-cultivation of the marine bacteria *Streptomyces* sp. and *Bacillus* sp. exhibited anticancer effects against human cancer cell lines. This suggested that co-cultivation of marine microorganisms might be a potential method for discovering novel bioactive microbial compounds (Shin *et al.*, 2018). Sran *et al.* (2019) found an actinobacterium, *Microbacterium aurantiacum* FSW25 from Rasthakaadu Beach (Tamil Nadu, India). This bacterium yielded a significant amount of an exopolysaccharide with remarkable rheological and antioxidant properties. Zhang *et al.* (2020) discovered a marine bacterium *Verrucosispora* sp. MS100137 isolated from sea sediments that produced a novel abyssomicin and six known abyssomicin and proximicin analogues. The novel molecule, together with two previously found compounds, showed strong antiviral activity. The metabolite C17-fengycin B, produced by *B. subtilis* 2H11 from a deep-sea environment, exhibited an inhibitory effect against the fungus *Fusarium solani* (Liu & Sun, 2021).

Fig 3: Antagonistic activity of C5

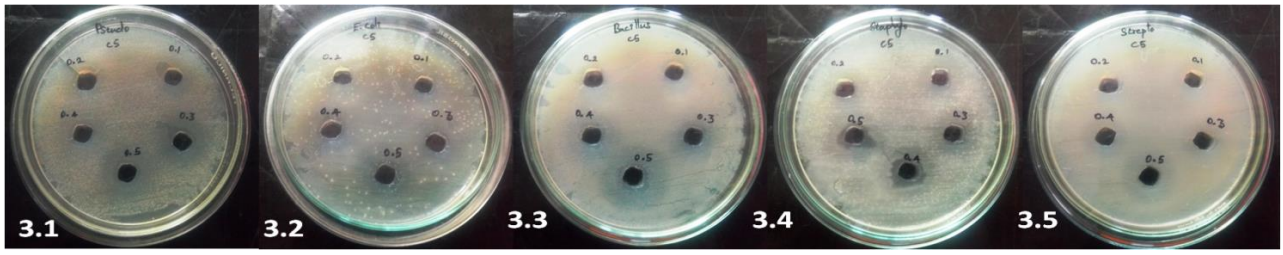

Fig 3.1- Antagonistic activity of C5 against *Pseudmonas* sp. at varying concentrations
Fig 3.2- Antagonistic activity of C5 against *Eschericia* sp. at varying concentrations
Fig 3.3- Antagonistic activity of C5 against *Bacillus* sp.at varying concentrations

Fig 3.4- Antagonistic activity of C5 against *Stapylococcus* sp. at varying concentrations
Fig 3.5- Antagonistic activity of C5 against *Streptococcus* sp.at varying concentrations

Fig 4: Antagonistic activity of M6

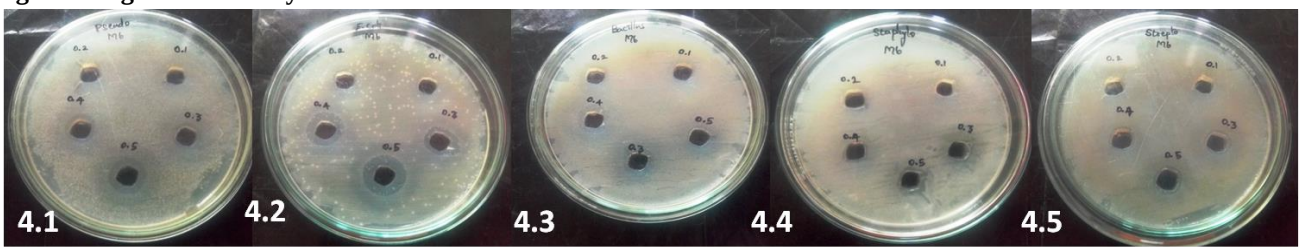

Fig 4.1- Antagonistic activity of M6 against *Pseudmonas* sp. at varying concentrations
Fig 4.2- Antagonistic activity of M6 against *Eschericia*sp. at varying concentrations
Fig 4.3- Antagonistic activity of M6 against *Bacillus* sp. at varying concentrations
Fig 4.4- Antagonistic activity of M6 against *Stapylococcus* sp. at varying concentrations
Fig 4.5- Antagonistic activity of M6 against *Streptococcus* sp.at varying concentrations

Table 4: Diameter of The Zone of Inhibition Measured for Antagonistic Activity of C5 Against Human Pathogens

Pathogen	Zone of inhibition (mm) (M±SE)				
	0.1ml	**0.2ml**	**0.3ml**	**0.4ml**	**0.5ml**
Pseudomonas aeruginosa	1±0.00	2±0.00	8.06±0.08	10.06±0.08	11±0.08
Escherichia coli	1.96±0.02	2.03±0.02	3.03±0.02	6±0.00	9.03±0.01
Bacillus cereus	0.06±0.02	0.1±0.08	4.9±0.02	11.23±0.09	13.3±0.02
Staphylococcus aureus	1.06±0.09	2.33±0.08	5.03±0.11	13±0.11	14.03±0.02
Streptococcus mutans	0.06±0.02	0.16±0.02	1.03±0.02	2.36±0.06	6.93±0.08

NOTE: The Zones of Inhibition are measured in terms of millimeters (mm) and expressed as Mean ±Standard Error (M ±SE)

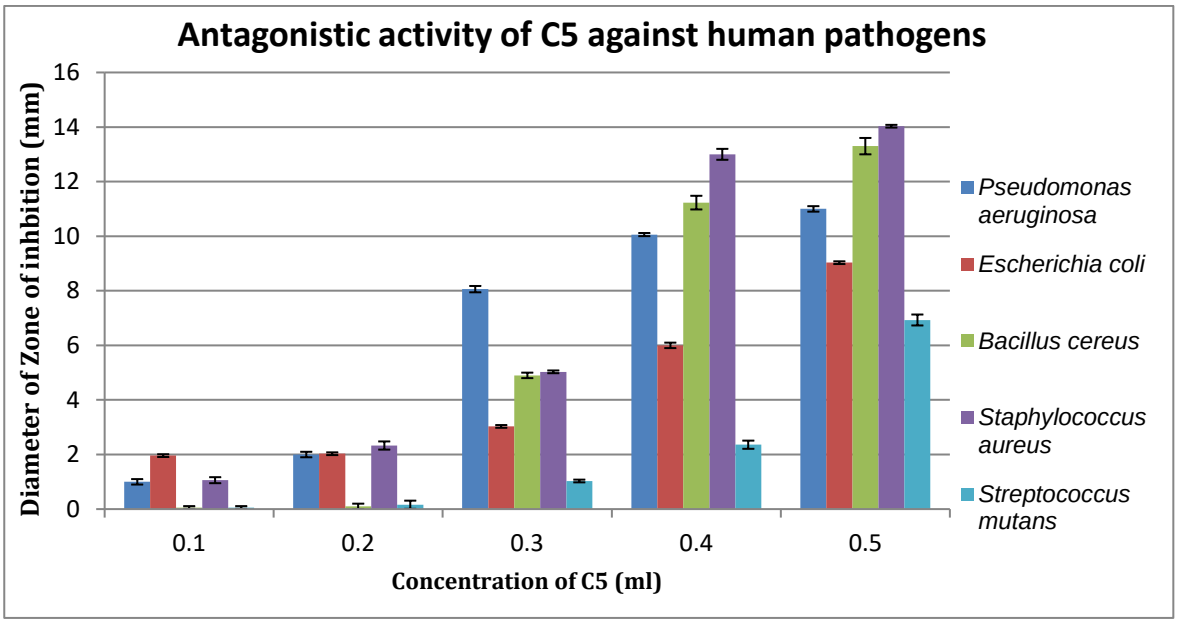

Figure 5: Antagonistic activity of C5 against human pathogens

Table 5: Diameter of The Zones of Inhibition Measured for Antagonistic Activity of M6 Against Human Pathogens

Pathogen	Zone of inhibition (mm) (M±SE)				
	0.1ml	0.2ml	0.3ml	0.4ml	0.5ml
Pseudomonas aeruginosa	0±0	0±0	4.96±0.15	6.96±0.15	11.8±0.15
Escherichia coli	0.0±0.05	0.9±0.05	1.03±0.05	4±0	4.9±0.01
Bacillus cereus	0.03±0.05	0.13±0.15	2.03±0.05	4.1±0.17	5.03±0.05
Staphylococcus aureus	1.1±0.17	1.16±0.15	2.93±0.20	6±0.2	6.03±0.05
Streptococcus mutans	0.03±0.05	0.06±0.05	4.06±0.05	5.06±0.11	4.86±0.15

NOTE: The Zones of Inhibition are measured in terms of millimeters (mm) and expressed as Mean ±Standard Error (M±SE)

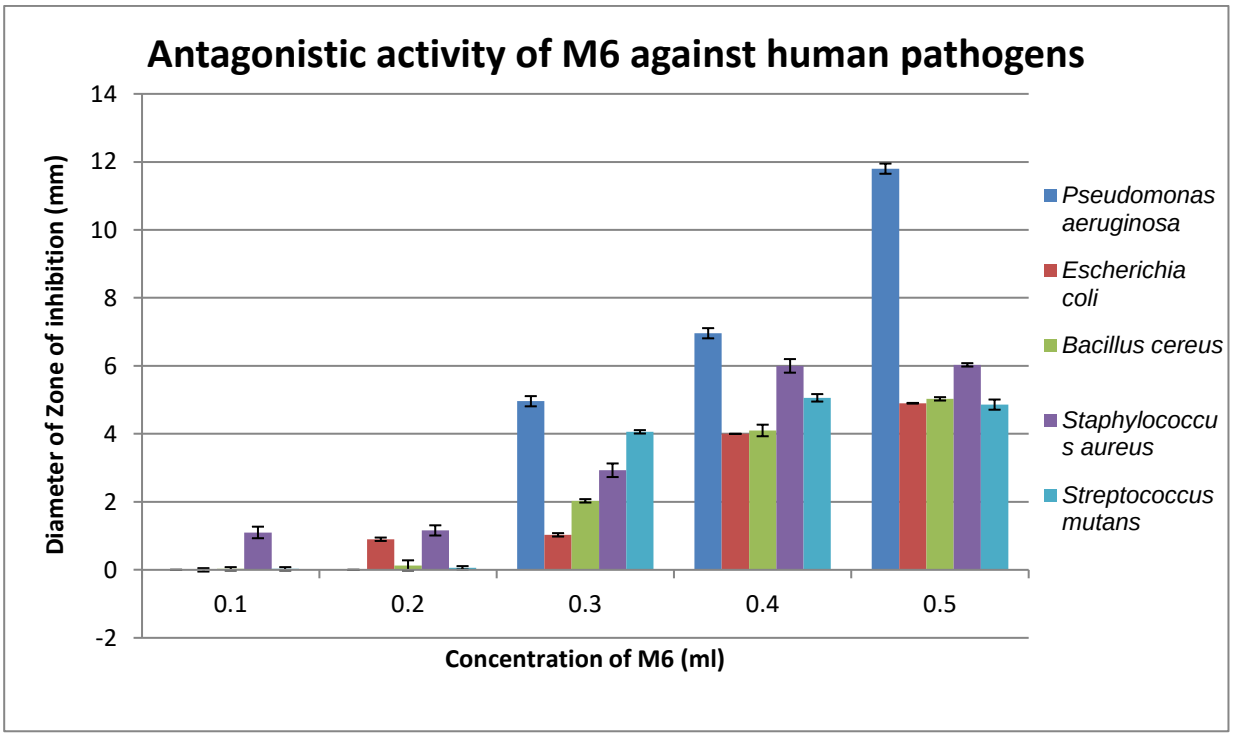

Figure 6: Antagonistic activity of M6 against human pathogens

GC-MS profiling

One of the primary methods for metabolite profiling that significantly contributes to the understanding of the metabolome is GC-MS-based metabolite profiling of biological samples. Since its inception as a significant technique for metabolite profiling, GC–MS is currently used routinely in functional genomic investigations of plants and microorganisms such as bacteria and fungus to screen for obvious or previously unknown metabolic characteristics (Kopka, 2006, Wani, *et al.*, 2010). In the present investigation, the culture supernatants of the selected isolates were subjected to thin layer chromatography. The eluted fractions were then analysed by Gas Chromatography Mass Spectrometry (GCMS). The results of the analysis showed the presence of the bioactive compounds as listed in the table 4.1 and 4.2. A majority of phenolic and benzene derivatives were found in the analysis. These compounds are reported to have profound antimicrobial properties (Yogeshwari *et al.*,2012). The cell free supernantant of C5 was found to contain 11 compounds namely 3-Methyl-4-isopropylphenol, Phenol, 2,4-bis(1,1-dimethylethyl)-, Cyclododecane, Propanoic acid, 3,3'-thiobis-, diethyl ester, Hexadecanoic acid, methyl ester, Pentadecanoic acid, ethyl ester, Methyl stearate, Octadecanoic acid, ethyl ester, Octadecane, Tris(tert-butyldimethylsilyloxy)arsane and Androst-5,15-dien-3ol acetate. The studies of the past have highlighted that 3-Methyl-4-isopropylphenol has anti-microbial (Li *et al.*,2013, Fujiki & Honda, 2020) and anti-oxidant properties (Ramos *et al.*,2018, Yogeswari *et al.*,2012, Salini *et al.*,2014, Das *et al.*, 2018), anti-fungal (Sabu R *et al.*,2014), anti-oxidant (Ajayi *et al.*,2011) and anti-tumor activity (Sujana *et al.*,2012, Panigrahi *et al.*,2014). Cyclododecane has reported to exhibit anti-microbial activity (Mordi *et al.*,2016), Hexadecanoic acid, methyl ester showed anti-oxidant, anti-inflammatory, anti-fungal, hypocholesterolemic, potent anti-microbial activity (Hema *et al.*,2011). The compound Methyl stearate has reported to act as GABA aminotransferase inhibitor, anti-inflammatory, intestinal lipid metabolism regulator, Gastrin inhibitor, anti-helminthic and antinociceptive (Adnan *et al.*, 2019). The compound Octadecanoic acid, ethyl ester has reported to show anti-microbial activity (Igwe & Okwu,2013). The biological activity of Octadecane is highlighted to have anti-fungal, anti-tumor activity, anti-bacterial (Gehan *et al.*,2009, Hsouna et al., 2011) and anti-microbial activities (Jasim *et al.*,2015).The compound Propanoic acid, 3, 3'-thiobis-, diethyl ester has reported to exhibit anti-inflammatory activity (Manilal *et al.*,2020).The biological activity of the compounds

Pentadecanoic acid ethyl ester, Tris(tert-butyldimethylsilyloxy)arsane and Androst-5,15-dien-3ol acetate remains unexplored.

The fraction eluted from the cell free supernatant of M6 showed the presence of 12 compounds such as Benzaldehyde, 4-methyl-, 1,2-Benzenediol, o-(4-methoxybenzoyl)-o'-(4-methylbenzoyl)-, 2-Butenedioic acid (Z)-, monododecyl ester, Phenol, 2,4-bis(1,1-dimethylethyl), Undecanoic acid, ethyl ester, Cyclododecane, Propanoic acid, 3,3'-thiobis-, diethyl ester, Pentadecanoic acid, 14-methyl-,methyl ester, 1,2-Benzenedicarboxylic acid, buyl 2-ethylhexyl ester, Hexadecanoic acid, ethyl ester, Methyl stearate and Octadecanoic acid, ethyl ester. Among these five compounds namely Phenol, 2,4-bis(1,1-dimethylethyl), Cyclododecane, Propanoic acid, 3,3'-thiobis-, diethyl ester , Methyl stearate and Octadecanoic acid were similar to the metabolites identified from C5. These compounds, exhibited the biological activities as mentioned above. The reason for this similarity could be that both the organisms have been isolated from marine environment. Previous investigations suggest that metabolites isolated from marine *Bacilli* are reported to be unusually different from those isolated from terrestrial origin (Jensen & Fencial *et al.*, 1994).

The compounds previously reported with anti-microbial properties were 1, 2-Benzenedicarboxylic acid, buyl 2-ethylhexyl ester (Osuntokun & Cristina *et al.*, 2019), Hexadecanoic acid, ethyl ester (Mohammadzadeh *et al.*, 2007) and Octadecanoic acidethyl ester (Gehan *et al.*, 2009). Specifically 2-Butenedioic acid (Z)-, monododecyl ester is anti-bacterial (Okwu & Ighodaro, 2009) and Pentadecanoic acid, 14-methyl-,methyl ester is an anti-fungal agent (Javaid *et al.*,2020). Anti-oxidant properties have been reported by the compounds 1, 2-Benzenediol, o-(4-methoxybenzoyl)-o'-(4-methylbenzoyl) (Yadav *et al.*, 2018) and 2-Butenedioic acid (Z)-, monododecyl ester (Shah *et al.*, 2014). The compound Undecanoic acid, ethyl ester has a previous record of tyrosinase inhibitor (Maamoun *et al.*, 2021).The biological activity of Benzaldehyde, 4-methyl- is not reported yet.

According to the results of the GC-MS analysis, the antibacterial activity demonstrated by these compounds might be attributed to the presence of individual antimicrobial compounds such as 3-Methyl-4-isopropylphenol, Phenol, 2,4-bis(1,1-dimethylethyl)-, Cyclododecane, Hexadecanoic acid, methyl ester, Octadecanoic acid, ethyl ester, Octadecane, 2-Butenedioic acid (Z)-, monododecyl ester, 1,2-Benzenedicarboxylic acid, buyl 2-ethylhexyl ester, Hexadecanoic acid, ethyl ester or to the combined action of all compounds. This theory, however, has to be investigated. The use of modern technologies such as nuclear magnetic resonance, bioassay-guided fractionation and Fourier-transform infrared spectroscopy could be used to predict the specific compound responsible for this antagonistic activity.

Fig 7 shows the TLC performed with the culture supernatants of C5 and M6. Lane 1 contains the sample from C5 and Lane 2 contains sample from M6. One prominent spot was observed in both the lanes indicated by an arrow mark.

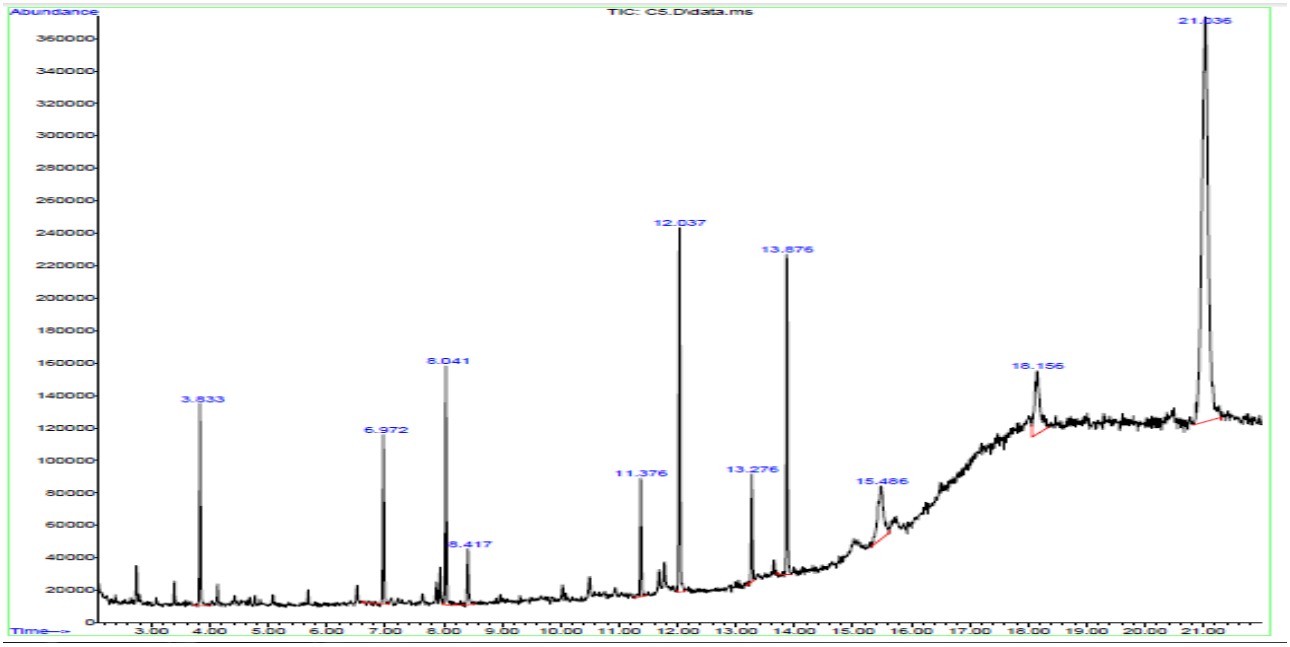

Fig 8.1 GCMS profile of C5

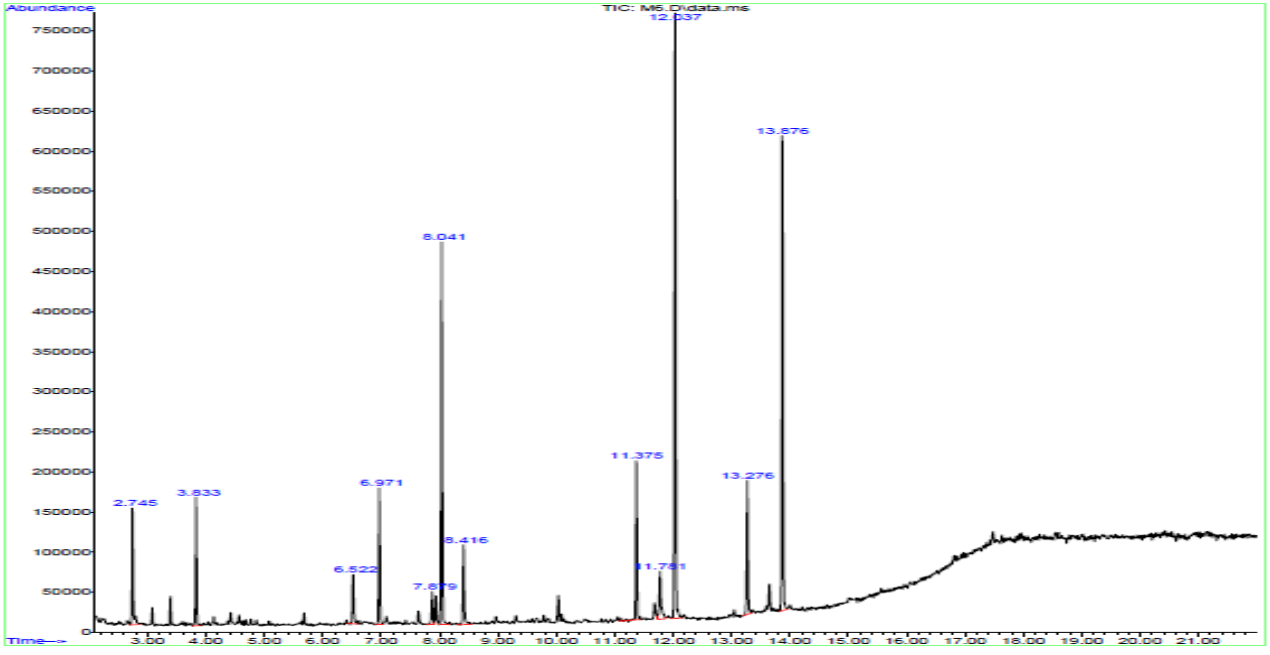

Fig 8.2 GCMS profile of M6

Figure 8: GCMS profiles of C5 & M6

Table 6.1 GCMS profile of C5

Peak number	Retention Time (mins)	Peak area	Peak area %	Match Quality (%)	Compounds	Reported activities
1	3.836	1646622	4.29	53	3-Methyl-4-isopropylphenol	Antimicrobial (Fujiki & Honda, 2020; Li *et al.*,2013), Antioxidant (Ramos *et al.*,2018)
2	6.975	1531056	3.99	95	Phenol, 2,4-bis(1,1-dimethylethyl)-	Antimicrobial (Das R*et al.*, 2018; Salini *et al.*,2014; Yogeswari *et al.*,2012), antifungal (Sabu R*et al.*,2014), antioxidant (Ajayi *et al.*,2011), antitumor (Panigrahi *et al.*,2014; Sujana *et al.*,2012)
3	8.044	2125530	5.54	98	Cyclododecane	Antimicrobial (Mordi *et al.*,2016)
4	8.412	699196	1.82	82	Propanoic acid, 3,3'-thiobis-, diethyl ester	Activity not explored
5	11.372	1129503	2.95	97	Hexadecanoic acid, methyl ester	Antifungal, Antioxidant, hypocholesterolemic nematicide, pesticide, antiandrogenic flavour, haemolytic, 5-Alpha reductase inhibitor, potent antimicrobial activity. (Hema *et al.*,2011)
6	12.034	3353387	8.75	91	Pentadecanoic acid, ethyl ester	Activity not explored
7	13.273	965194	2.52		Methyl stearate	GABA aminotransferase inhibitor Anti-inflammatory, intestinal Lipid metabolism regulator Gastrin inhibitor Antihelmintic (Nematodes) Antinociceptive (Adnan *et al.*,2019)
8	13.878	3023561	7.88	95	Octadecanoic acid, ethyl ester	Antimicrobial (Igwe & Okwu,2013).
9	15.485	2284077	5.96	83	Octadecane	Antifungal, antitumor activity, antibacterial (Gehan *et al.*,2009; Hsouna et a., 2011) Antimicrobial (Jasim *et al.*,2015)
10	18.151	2542271	6.63	43	Tris(tert-butyldimethylsilyloxy)arsane	Activity not explored
11	21.035	19045675	49.67	59	Androst-5,15-dien-3ol acetate	Activity not explored

Fig 9 Mass spectrum of individual compounds identified from the cell free supernatant of C5

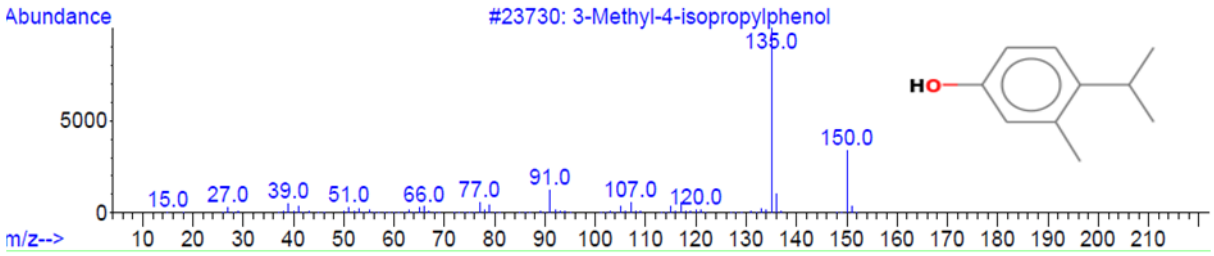

Fig 9.1 Mass spectrum of 3-Methyl-4-isopropylphenol

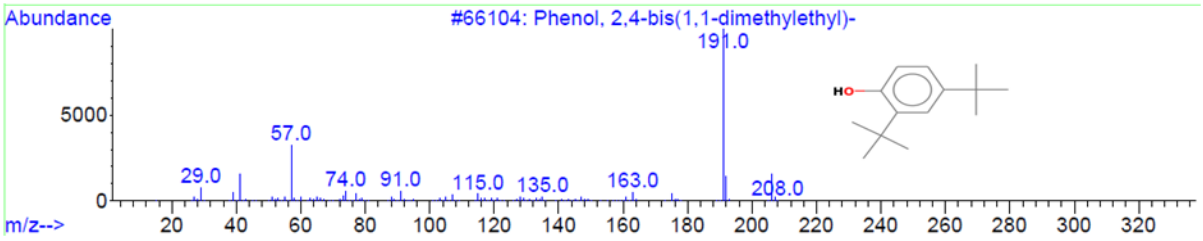

Fig 9.2 Mass spectrum of Phenol, 2,4-bis(1,1-dimethylethyl)-

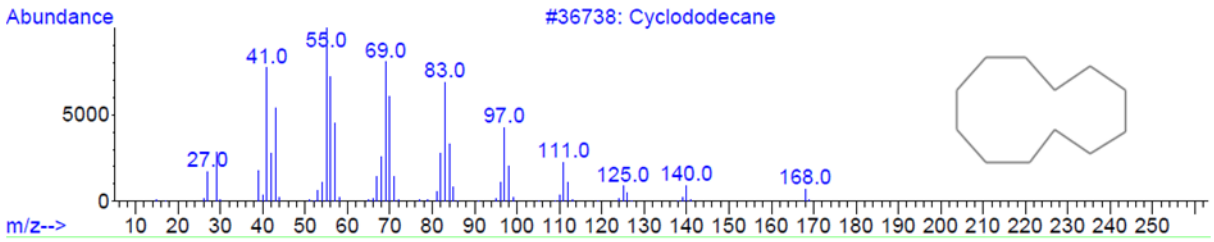

Fig 9.3 Mass spectrum of Cyclododecane

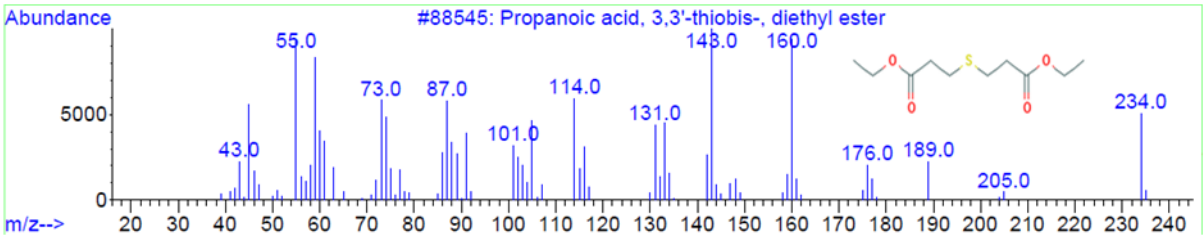

Fig 9.4 Mass spectrum of Propanoic acid, 3,3'-thiobis-, diethyl ester

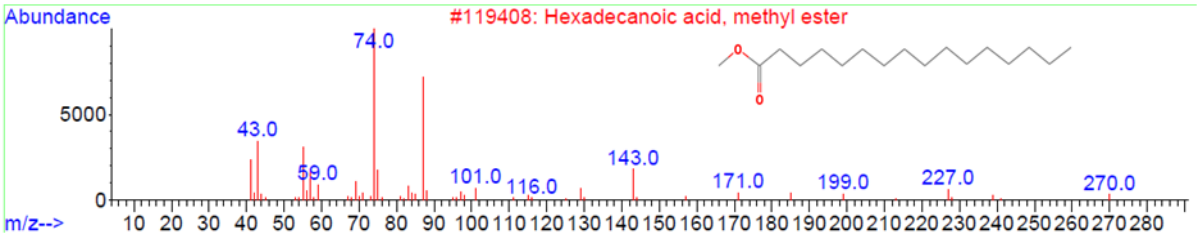

Fig 9.5 Mass spectrum of Hexadecanoic acid, methyl ester

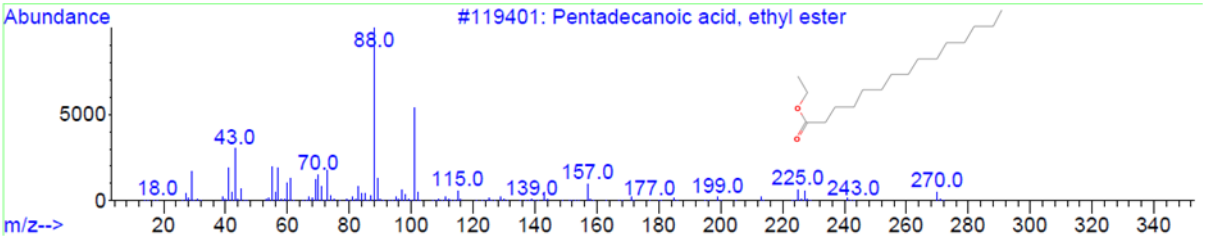

Fig 9.6 Mass spectrum of Pentadecanoic acid, ethyl ester

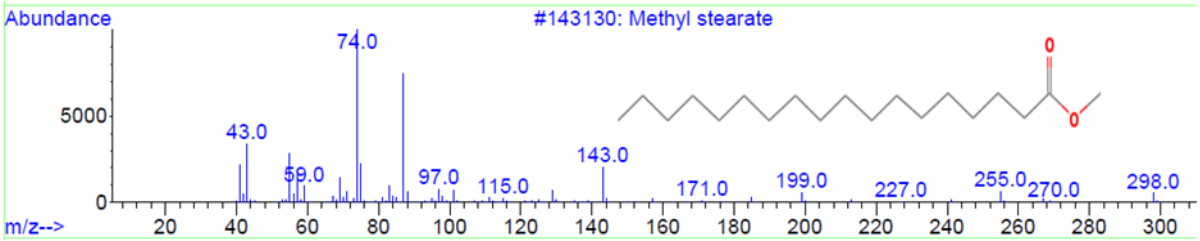

Fig 9.7 Mass spectrum of Methyl stearate

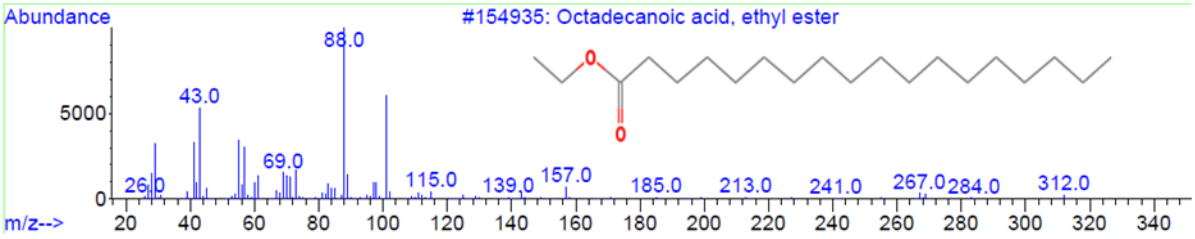

Fig 9.8 Mass spectrum of Octadecanoic acid, ethyl ester

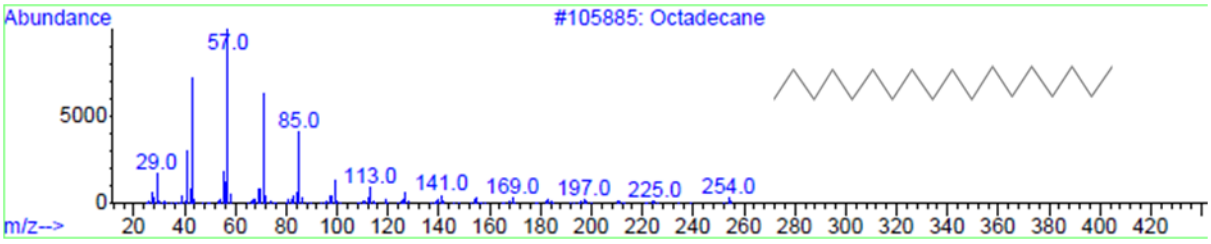

Fig 9.9 Mass spectrum of Octadecane

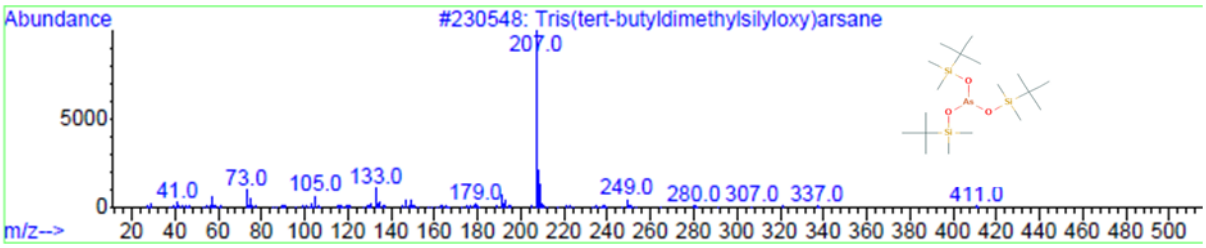

Fig 9.10 Mass spectrum of Tris(tert-butyldimethylsilyloxy)arsane

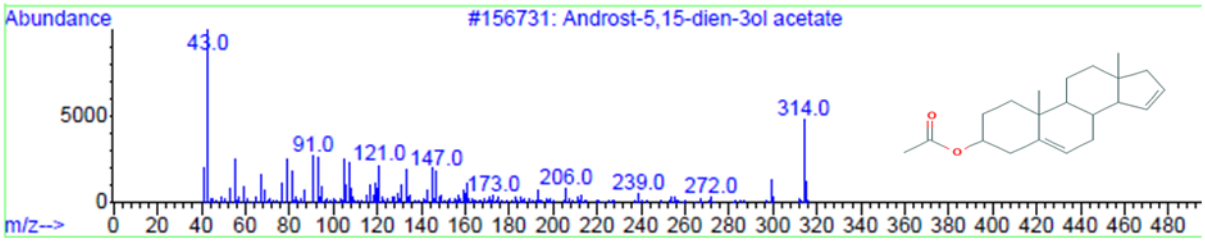

Fig 9.11 Mass spectrum of Androst-5,15-dien-3ol acetate

Table 6.2 GCMS profiles of M6

Peak number	Retention Time (min)	Peak area	Peak area %	Match Quality (%)	Compounds	Reported activities
1	2.749	2146914	4.92	97	Benzaldehyde, 4-methyl-	Activity not explored
2	3.836	2056445	4.71	50	1,2-Benzenediol, o-(4-methoxybenzoyl)-o'-(4-methylbenzoyl)-	Anti-oxidant activity (Yadav *et al.*,2018)
3	6.521	1024390	2.35	91	2-Butenedioic acid (Z)-, monododecyl ester	Anti-bacterial, (Okwu & Ighodaro, 2009), Anti-oxidant (Shah et al,2014)
4	6.975	2400716	5.50	97	Phenol, 2,4-bis(1,1-	Antimicrobial (Das R*et*

					dimethylethyl)	*al.*, 2018; Salini *et al.*,2014; Yogeswari *et al.*,2012), antifungal (Sabu R*et al.*,2014), antioxidant (Ajayi *et al.*,2011), antitumor (Panigrahi *et al.*,2014; Sujana *et al.*,2012)
5	7.883	668104	1.53	72	Undecanoic acid, ethyl ester	Tyrosinase inhibitor (Maamoun *et al.*,2021)
6	8.044	6629246	15.20	96	Cyclododecane	Antimicrobial Mordi *et al.*,2016)
7	8.412	1595813	3.66	99	Propanoic acid, 3,3'-thiobis-, diethyl ester	Anti-inflammatory (Manilal *et al.*,2020)
8	11.372	2927096	6.71	97	Pentadecanoic acid, 14-methyl-,methyl ester	Anti-fugal (Javaid *et al.*,2020)
9	11.778	1324523	3.04	86	1,2-Benzenedicarboxylic acid, buyl 2-ethylhexyl ester	Antimicrobial (Osuntokun & Cristina ,2019)
10	12.034	11137214	25.53	97	Hexadecanoic acid, ethyl ester	Antimicrobial (Mohammadzadeh *et al.*,2007).
11	13.272	2420833	5.55	98	Methyl stearate	GABA aminotransferase inhibitor Anti-inflammatory, intestinal Lipid metabolism regulator Gastrin inhibitor Antihelmintic (Nematodes) Antinociceptive (Adnan *et al.*,2019)
12	13.878	9285940	21.29	98	Octadecanoic acid, ethyl ester	Antimicrobial (Gehan *et al.*,2009)

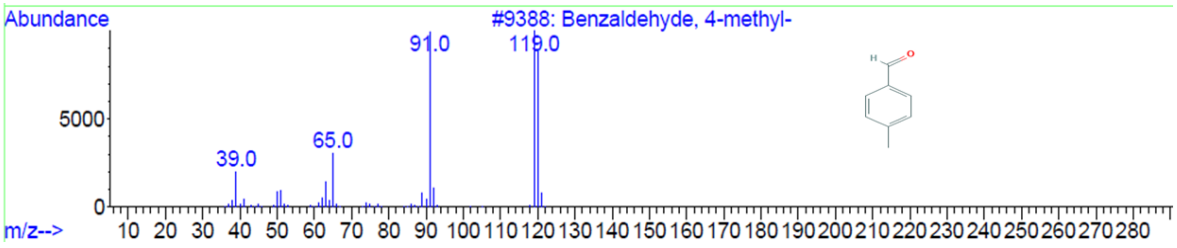

Fig 10.1Mass spectrum and chemical structure of Benzaldehyde, 4-methyl-

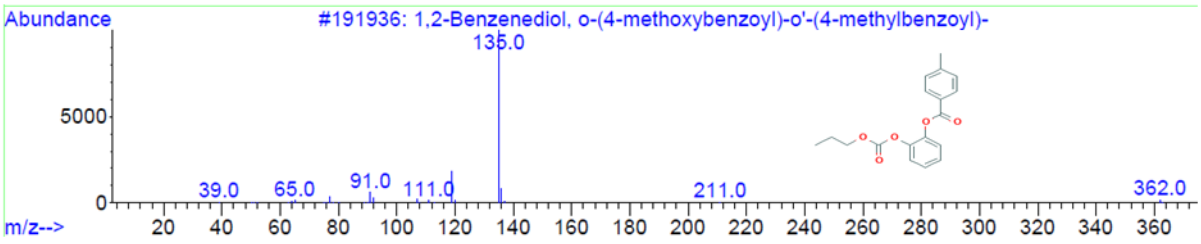

Fig 10.2 Mass spectrum of 1,2-Benzenediol, o-(4-methoxybenzoyl)-o'-(4-methylbenzoyl)-

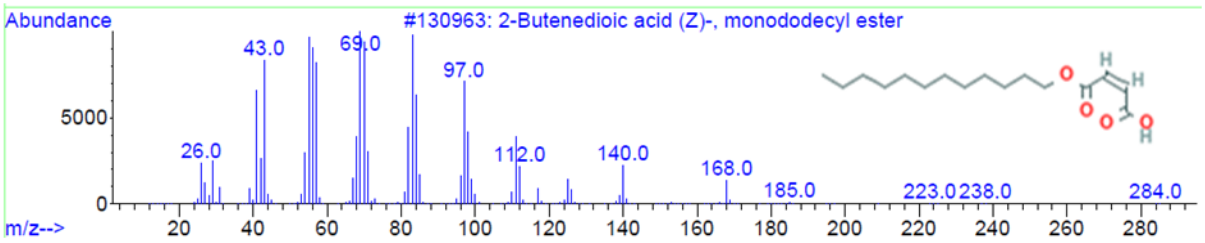

Fig 10.3 Mass spectrum of 2-Butenedioic acid (Z)-, monododecyl ester

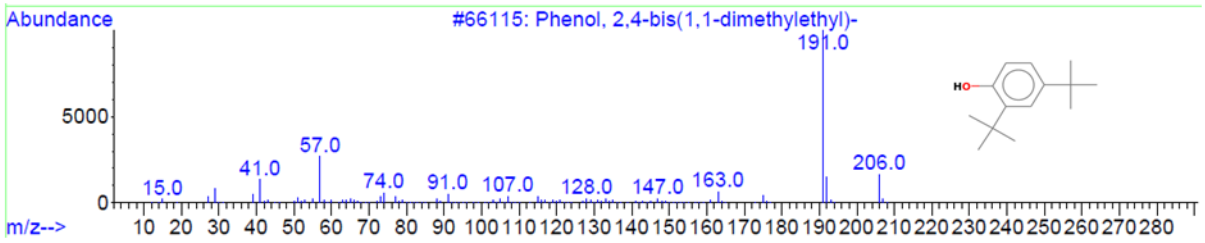

Fig 10.4 Mass spectrum of Phenol, 2,4-bis(1,1-dimethylethyl)

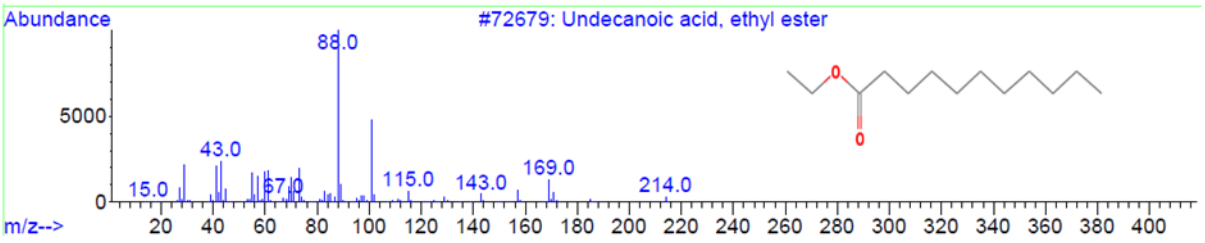

Fig 10.5 Mass spectrum of Undecanoic acid, ethyl ester

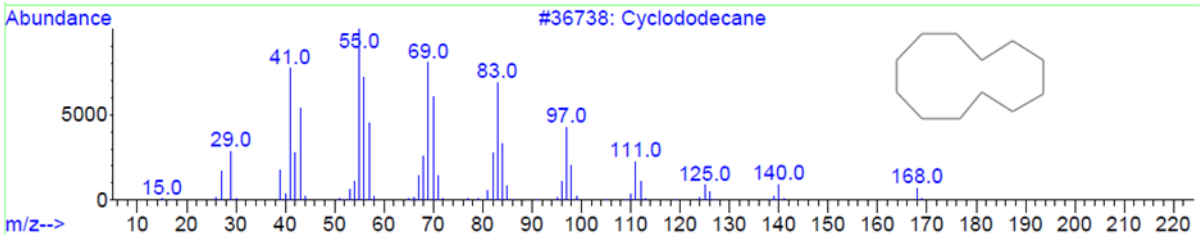

Fig 10.6 Mass spectrum of Cyclododecane

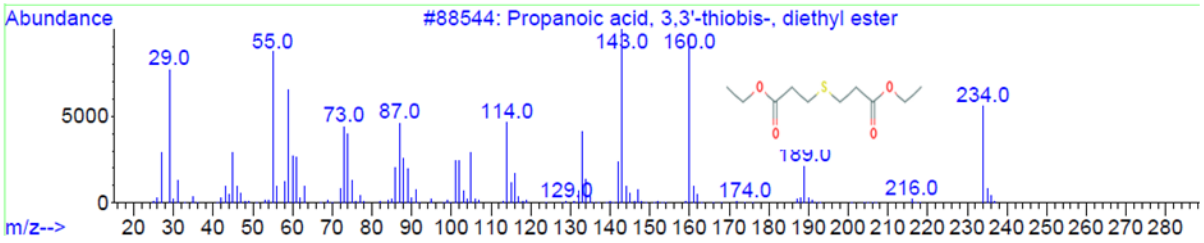

Fig 10.7 Mass spectrum of Propanoic acid, 3,3'-thiobis-, diethyl ester

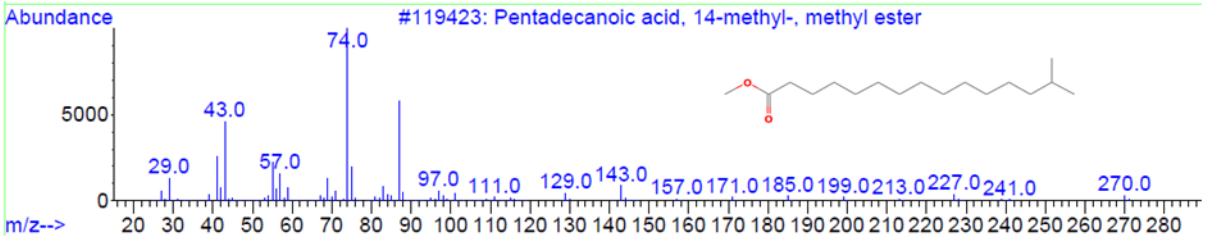

Fig 10.8 Mass spectrum of Pentadecanoic acid, 14-methyl-,methyl ester

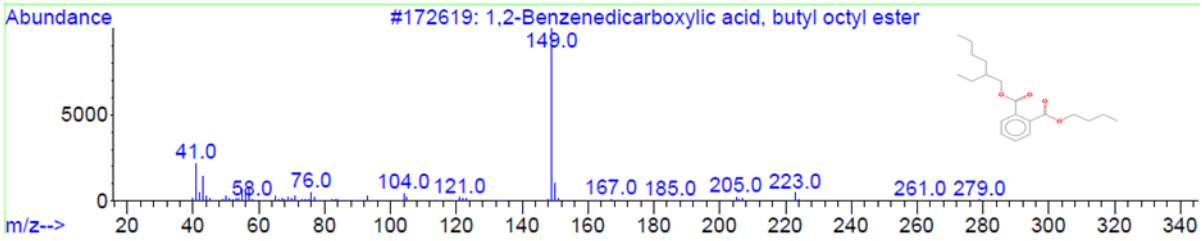

Fig 10.9 Mass spectrum of 1,2-Benzenedicarboxylic acid, buyl 2-ethylhexyl ester

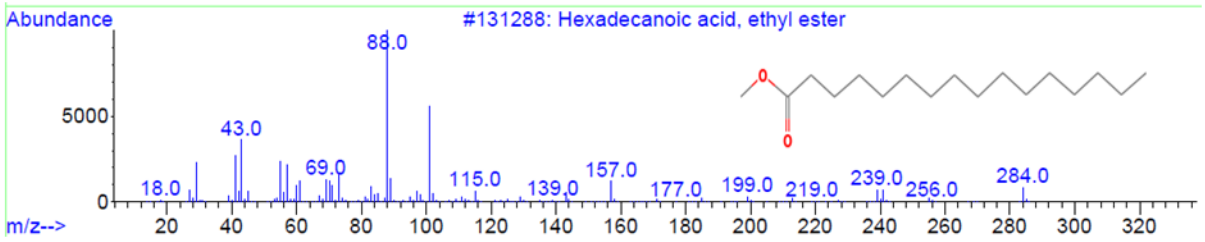

Fig 10.10 Mass spectrum of Hexadecanoic acid, ethyl ester

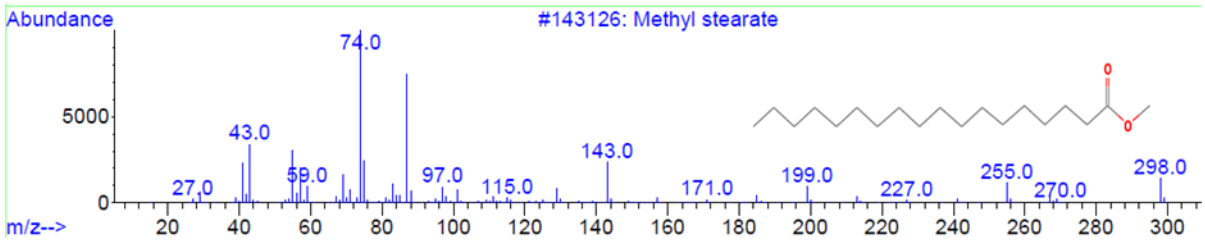

Fig 10.11 Mass spectrum of Methyl stearate

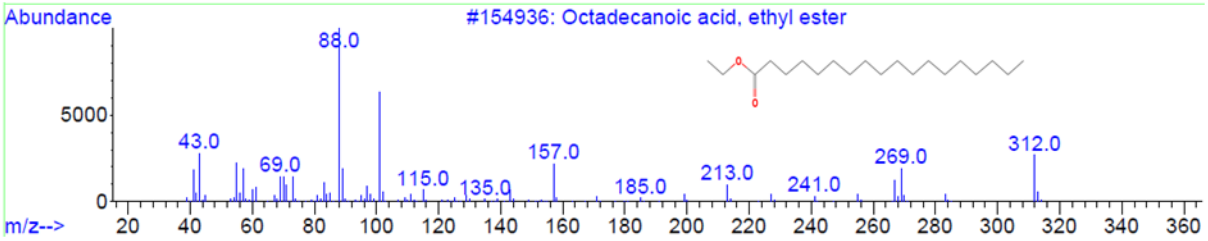

Fig 10.12 Mass spectrum of Octadecanoic acid, ethyl ester

Figure10 Mass spectrum and chemical structure of individual compounds identified from the cell free supernatant of M6

Phylogenetic tree construction

To identify the species of the two bacterial isolates, 16S rRNA gene sequencing was performed. The organism C5 was identified to be *Bacillus marisflavi* and M6 was identified to be *Bacillus flexus*. The sequences of C5 and M6 have been deposited in GenBank under accession number MN368726 and MN368862 respectively. The molecular Phylogenetic tree was constructed by Maximum Likelihood method as shown in Figure 17. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). The tree with the highest log likelihood (-2237.17) is shown in Fig.3. The percentage of trees in which the associated taxa were clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances, estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. All positions with less than 95% site coverage were

eliminated; i.e. fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1396 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

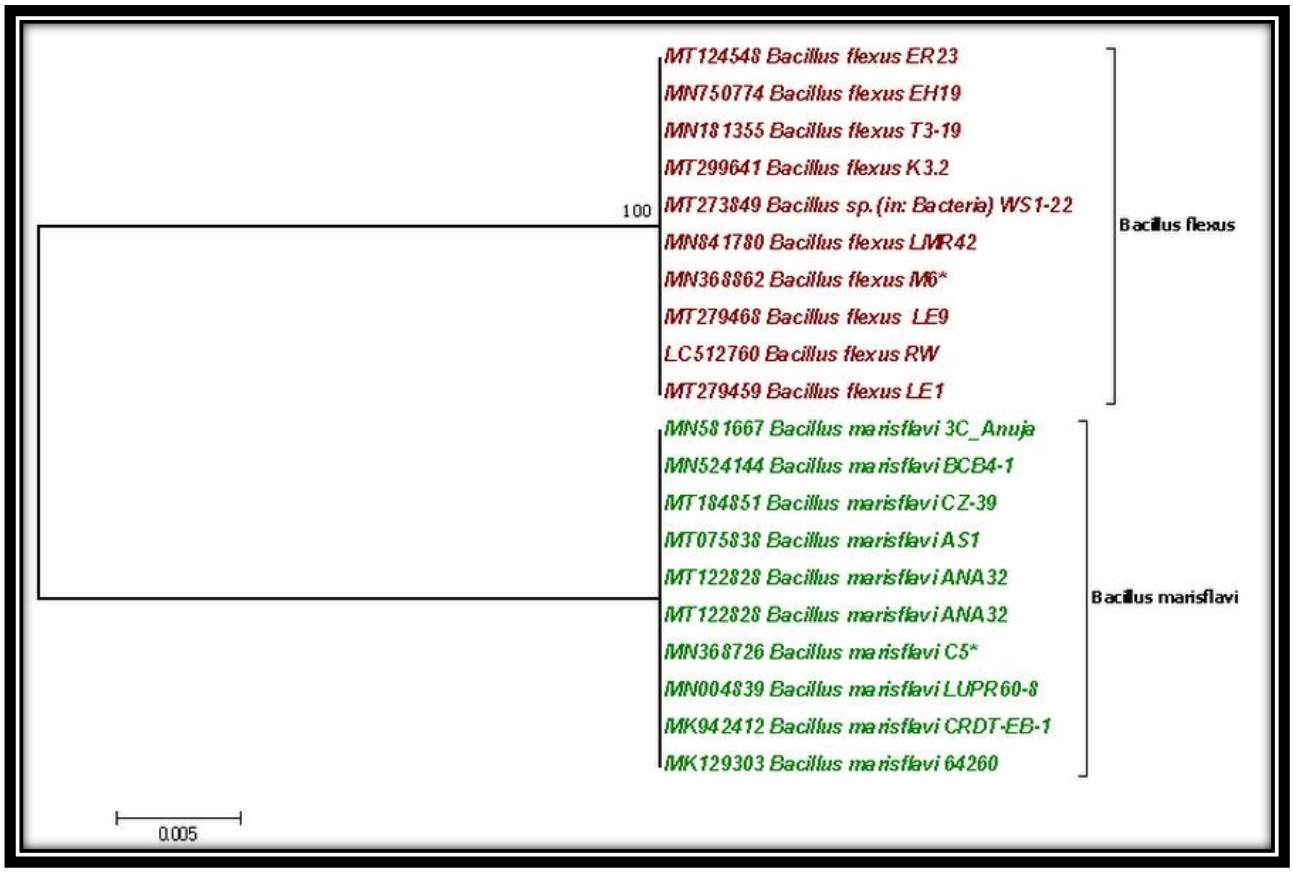

Fig 11. Molecular Phylogenetic analysis by Maximum Likelihood method

Conclusion

Bacillus species from marine environments produce structurally diverse natural compounds as a result of complicated biochemical processes. Because the living environment for marine microorganisms is exceedingly difficult, they create a massive number of bioactive chemicals to ensure their survival. There is always a continuous demand for the search for new pharmaceutical products to meet the needs of the growing population. Lipopeptides, polysaccharides, macrolactones, fatty acids, polyketides, antiviral compounds, antibiotics, and pigments are among the structurally varied types of secondary metabolites. Some of these bioactive chemicals have considerable potential for development of useful pharmaceutical and agrochemical applications. As per the literature available for *B. marisflavi* and *B. flexus*, a very less amount of work has been done with these two microbial strains so far. Hence an intensive study with modern molecular techniques could be applied to study the structure and mode of action of these novel metabolites. Thus, the metabolites obtained from these microbes can be used to meet the needs of a growing population with a reduced environmental hazard. In conclusion, it is exposed that marine water samples of India have a potent source of bacteria.

Authors' contribution

Thelma J: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft. **Balasubramanian C:** Writing - Review & Editing, Project administration

Acknowledgements

Authors thank the Management of Thiagarajar College, Madurai for providing all necessary facilities to carry out the research successfully.

Conflict of interests

The authors declare that there is no conflict of interest

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors

References

Adnan, M., Nazim Uddin Chy, M., Mostafa Kamal, A. T. M., Azad, M., Paul, A., Uddin, S., Cho, D. (2019). Investigation of the Biological Activities and Characterization of Bioactive Constituents of Ophiorrhiza rugosa var. prostrata (D.Don) & Mondal Leaves through In Vivo, In Vitro, and In Silico Approaches. *Molecules*, *24*(7), 1367. doi:10.3390/molecules24071367.

Ajayi, G.O. & Olagunju J, Oladipo A & Martins O C. (2011) . Gas chromatography-mass spectrometry analysis and phytochemical screening of ethanolic root extract of Plumbago zeylanica, Linn. Journal of Medicinal Plant Research. 5:1756-1761.

Al-Dhabi, N. A., Mohammed Ghilan, A. K., Esmail, G. A., Valan Arasu, M., Duraipandiyan, V., & Ponmurugan, K. (2019). Bioactivity assessment of the Saudi Arabian Marine Streptomyces sp. Al-Dhabi-90, metabolic profiling and its in vitro inhibitory property against multidrug resistant and extended-spectrum beta-lactamase clinical bacterial pathogens. *Journal of infection and public health*, *12*(4), 549–556. https://doi.org/10.1016/j.jiph.2019.01.065

Ash, C., Farrow, J.A.E., Wallbanks, S., Collins, M.D. (1991) Phylogenetic heterogeneity of the genus *Ballicus* revealed by comparative analysis of the small-subunit-ribosomal RNA, *Lettersin Applied Microbiology*. 13, 202–206.

Berrue, F., Ibrahim, A., Boland, P. & Kerr, R. (2009). Newly isolated marine Bacillus pumilus (SP21): A source of novel lipoamides and other antimicrobial agents. *Pure and Applied Chemistry*, *81*(6), 1027-1031. https://doi.org/10.1351/PAC-CON-08-09-25

Cappuccino JG, Sherman N. Microbiology A Laboratory Manual.5th Edition. Pearson Education Inc. San Francisco, 2002, pp: 63-65.

Chellaram, C., Gnanambal, K.M.E. and Edward, J.K.P. Antibacterial activity of the winged oyster Pteria chinensis (Pterioida: Pteridae). (2004) Indian Journal of Marine Science, 33(4): 369-372.

Chen, X. H., Scholz, R., Borriss, M., Junge, H., Mögel, G., Kunz, S., & Borriss, R. (2009). Difficidin and bacilysin produced by plant-associated Bacillus amyloliquefaciens are efficient in controlling fire blight disease. *Journal of biotechnology*, *140*(1-2), 38–44. https://doi.org/10.1016/j.jbiotec.2008.10.015

Claus, D. and Berkeley, R.C.W. (1986) Genus Bacillus Cohn, 1872. In: Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt. J.G., Eds., Bergey's Manual of Systematic Bacteriology, The Williams & Wilkins Co., Baltimore, 2, 1105-1139.

Cubeta, M.A., Hartman, G.L. and Sinclair, J.B (1985) Interaction between *Bacillus subtilis* and fungi associated with soybean seeds. *Plant Disease*,69: 506-509.

Das, R., Romi, W., Das, R, Sarma H, Thakur, D. (2018) Antimicrobial potentiality of actinobacteria isolated from two microbiologically unexplored forest ecosystems of Northeast India. *BMC Microbiol* , **18:** 71 https://doi.org/10.1186/s12866-018-1215-7

Ferreira, J.H.S., Matther, F.N. and Thomas, A.C (1991) Biological control of *Eutypalata* on grapevine by an antagonistic strain of *Bacillus subtilis. Phytopathology*, 81: 283-287.

Fravel, D.R. (1988) Role of antibiosis in the biocontrol of plant diseases. *Annual Review of Phytopathology* , 26: 75-91.

Fujiki, M., & Honda, M. (2020). The investigation of synergistic activity of protamine with conventional antimicrobial agents against oral bacteria. *Biochemical and biophysical research communications*, *523*(3), 561–566. https://doi.org/10.1016/j.bbrc.2020.01.002

Fusetani, N., Ejima, D., Matsunaga, S., Hashimoto, K., Itagaki, K., Akagi, Y., Taga, N., & Suzuki, K. (1987). 3-Amino-3-deoxy-D-glucose: an antibiotic produced by a deep-sea bacterium. *Experientia*, *43*(4), 464–465. https://doi.org/10.1007/BF01940457

Galaviz-silva, L., Iracheta-Villarreal, J.M., Molina-Garza, Z.J. (2018) Bacillus and Virgibacillus strains isolated from three Mexican coasts antagonize Staphylococcus aureus and Vibrio parahaemolyticus, *FEMS Microbiology Letters*, 365(19), fny202 https://doi.org/10.1093/femsle/fny202

Gehan, M.A., Hanan, A.E., Hassan, A.H.I., and Okbah, M.A. (2009) Marine natural products and their potential applications as antiinfective agents. World Sciences Journal, 7(7): 872-880.

Gerard, J. M., Haden, P., Kelly, M. T., & Andersen, R. J. (1999). Loloatins A-D, cyclic decapeptide antibiotics produced in culture by a tropical marine bacterium. *Journal of natural products*, *62*(1), 80–85. https://doi.org/10.1021/np980219f

Hema R., Kumaravel, S., and Alagusundaram, K., (2011) GC/MS Determination of Bioactive Components of *Murraya koenigii*. Journal of American Science, 7(1):80-83

Hsouna, A. B., Trigui, M., Mansour, R. B., Jarraya, R. M., Damak, M., & Jaoua, S. (2011). Chemical composition, cytotoxicity effect and antimicrobial activity of Ceratonia siliqua essential oil with preservative effects against Listeria inoculated in minced beef meat. *International journal of food microbiology*, *148*(1), 66–72. https://doi.org/10.1016/j.ijfoodmicro.2011.04.028

Igwe, O.U., Okwu, D.E., (2013) GC-MS evaluation of bioactive compounds and antibacterial activity of the oil fraction from the seeds of *Brachystegia eurycoma* (HARMS) *Asian Journal of Plant Science and Research*, 3(2),47-54.

Ivanova, E., Nicolau, D., Yumoto, N., Taguchi, T., Okamoto, K., Tatsu, Y., Yoshikawa, S., (1998) Impact of conditions of cultivation and adsorption on antimicrobial activity of marine bacteria. *Marine Biology* **130,** 545–551. https://doi.org/10.1007/s002270050275

Jasim, H., Hussein, A.O., Hameed, I.H., & Kareem, M.A, (2015) Characterization of alkaloid constitution and evaluation of antimicrobial activity of *Solanum nigrum* using gas chromatography mass spectrometry (GC-MS). *Journal of Pharmacognosy and Phytotherapy*, 7(4), 56-72.

Javaid, A., Afzal, R., Shoaib, A., (2020) Biological management of southern blight of chili by *Penicillium oxalicum* and leaves of *Eucalyptus citriodora*. International Journal of Agriculture and Biology, 23: 93–102.

Jensen, P. R., & Fenical, W. (1994). Strategies for the discovery of secondary metabolites from marine bacteria: ecological perspectives. *Annual review of microbiology*, *48*, 559–584. https://doi.org/10.1146/annurev.mi.48.100194.003015

Jensen, P.R., Fencial, W., Harvell, C.D., Wirtz, K. (1996) Antimicrobial activity of extracts of Caribbean gorgonian corals. *Marine Biology* **125,** 411–419. https://doi.org/10.1007/BF00346321

Kopka J. (2006). Current challenges and developments in GC-MS based metabolite profiling technology. *Journal of biotechnology*, *124*(1), 312–322. https://doi.org/10.1016/j.jbiotec.2005.12.012

Krawczyk, A. O., de Jong, A., Eijlander, R. T., Berendsen, E. M., Holsappel, S., Wells-Bennik, M. H., & Kuipers, O. P. (2015). Next-Generation Whole-Genome Sequencing of Eight Strains of Bacillus cereus, Isolated from Food. *Genome announcements*, *3*(6), e01480-15. https://doi.org/10.1128/genomeA.01480-15

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular biology and evolution*, *33*(7), 1870–1874.https://doi.org/10.1093/molbev/msw054

Li, C., Huang, J. G., Lu, T. L., & He, R. J. (2013) GC-MS analysis of chemical components of volatile oil from *Viburnm schensianum* leaf and their antibacterial activity,

Journal of Chinese medicinal materials, *36*(12), 1962–1966.

Li, J., Wang, Y., Hao, X., Li, S., Jia, J., Guan, Y., Peng, Z., Bi, H., Xiao, C., Cen, S., & Gan, M. (2019). Broad-Spectrum Antiviral Natural Products from the Marine-Derived *Penicillium* sp. IMB17-046. *Molecules (Basel, Switzerland)*, *24*(15), 2821. https://doi.org/10.3390/molecules24152821

Liu, W., Sun, C., (2021) C_{17}-fengycin B, produced by deep-sea-derived *Bacillus subtilis*, possessing a strong antifungal activity against *Fusarium solani*. *Journal of Oceanology and Limnology*
https://doi.org/10.1007/s00343-020-0215-2

Liu, Y., Lai, Q., Du, J., & Shao, Z. (2017). Genetic diversity and population structure of the Bacillus cereus group bacteria from diverse marine environments. *Scientific reports*, *7*(1), 689. https://doi.org/10.1038/s41598-017-00817-1

Loeffler, W., Tschen, J.S.M., Vanittanatkcom, N., Kugler, M., Knorpp, E., Hsieh, T.F. and Wu, T.G (1986). Antifungal effects of bacilysin and fengymycin from *Bacillus subtilis* F-29-3. A comparison with activities of other *Bacillus* antibiotics. *Journal of Phytopathology* ,115, 204-213.

Manilal, A., Sabu, K. R., Shewangizaw, M., Aklilu, A., Seid, M., Merdikios, B., & Tsegaye, B. (2020). *In vitro* antibacterial activity of medicinal plants against biofilm-forming methicillin-resistant *Staphylococcus aureus*: efficacy of *Moringa stenopetala* and *Rosmarinus officinalis* extracts. *Heliyon*, *6*(1), e03303. https://doi.org/10.1016/j.heliyon.2020.e03303

Mohammadzadeh, S., Shariatpanahi, M., Hamedi, M., Ahmadkhaniha, R., Samadi, N., Ostad, S.N., (2007) Chemical composition, oral toxicity and antimicrobial activity of Iranian propolis, *Food Chemistry*, 103(4), 1097-1103. https://doi.org/10.1016/j.foodchem.2006.10.006

Mordi, R.C, Fadiaro, A.E., Owoeye, T.F., Olanrewaju, I.O., Uzoamaka, G.C., and Olorunshola, S.J (2015) Identification by GC-MS of the Components of Oils of Banana Peels Extract, Phytochemical and Antimicrobial Analyses, Research Journal of Phytochemistry 10 (1), 39-44.
DOI:https://dx.doi.org/10.3923/rjphyto.2016.39.44

Okami, Y., Kurasawa, S., Hirose, Y., (1980) A new glucanase produced by marine *Bacillus*. *Agricultural and Biological Chemistry*, 44:1191–1192.

Okwu D.E, Ighodaro, B.U. (2009). GC-MS evaluation of the bioactive compounds and antibacterial activity of the oil fraction from the stembarks of dacryodes edulis g. Don lam. *International Journal of Drug Development and Research*, 1.

Osuntokun O.T., Cristina, G.M., (2019) Bio-guided isolation, chemical purification, identification, antimicrobial and synergistic efficacy of extracted essential oils from stem bark extract of *Spondias mombin* (Linn). *International Journal of Molecular Biology* Open Access. 4(4),135–143. DOI: 10.15406/ijmboa.2019.04.00110

Panigrahi, S, Muthuraman, M.S., Natesan, R., Pemaiah, B., (2014) Anticancer activity of Ethanolic extract of *Solanum torvum* SW. *International Journal of Pharmacy and Pharmaceutical Sciences*, 6,93-98.

Piewngam, P., Zheng, Y., Nguyen, T. H., Dickey, S. W., Joo, H. S., Villaruz, A. E., Glose, K. A., Fisher, E. L., Hunt, R. L., Li, B., Chiou, J., Pharkjaksu, S., Khongthong, S., Cheung, G., Kiratisin, P., & Otto, M. (2018). Pathogen elimination by probiotic Bacillus via signalling interference. *Nature*, *562*(7728), 532–537.
https://doi.org/10.1038/s41586-018-0616-y

Priest, F.G, Systematics and ecology of Bacillus. In: Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology and Molecular Genetics (Hoch, J.A. and Losick, R., Eds.), American Society for Microbiology (ASM), Washington, DC, 1993, pp.3-16.

Ramos, I.F.M, Estrella, H.E.Z, Vargas, J.A.R., Erosa, F.E., Qjeda, N.G., Sosa, K.G., Roderiquez, L.M.P (2018), Bioactive dicaffeoylquinic acid derivatives from the root extract of *Calea urticifolia*. *Rev. Bras. Farmacogn*, **28,** 339–343 https://doi.org/10.1016/j.bjp.2018.01.010

Sabu, R., Soumya, K. R., & Radhakrishnan, E. K. (2017). Endophytic Nocardiopsis sp. from Zingiber officinale with both antiphytopathogenic mechanisms and antibiofilm activity against clinical isolates. *3 Biotech*, *7*(2), 115. https://doi.org/10.1007/s13205-017-0735-4

Sagar, A., Gaurab, K., Sunil, P. (2015) Microbial diversity in Freshwater and Marine Environment, *Nepal Journal of Biotechnology*, 3(1), 68-70.

Salah Maamoun, H., Rabie, G. H., Shaker, I., A Alaidaroos, B., & El-Sayed, A. (2021). Biochemical Properties of Tyrosinase from *Aspergillus terreus* and *Penicillium copticola*; Undecanoic Acid from *Aspergillus flavus*, an Endophyte of *Moringa oleifera*, Is a Novel Potent Tyrosinase Inhibitor. *Molecules (Basel, Switzerland)*, *26*(5), 1309. https://doi.org/10.3390/molecules26051309

Salini, T.S., Divakaran, D., Rebello, S., Shanavas, J., (2014) Antimicrobial and immunomodulatory potential of endophytic fungus *Fusarium solani* isolated from *Withania somnifera*. World Journal of Pharmaceutical Research, 3,879-890.

Seifert, K.A., Hamilton, W.E., Breuil, C. and Best, M., (1987) Evaluation of *Bacillus subtilis* C186 as a potential biological control of sapstain and mould on unseasoned lumber, *Canadian Journal of Microbiology*, 33, 1102-1107.

Sharp, R.J. , Riley, P.W., White, D., (1992) Heterotrophic Thermophilic Bacilli. In: Thermophilic Bacteria. Kristjanssono, J.K.,, Ed., CRC Press, Inc.: Boca Raton , pp. 19–50.

Shah, M. D., Yong, Y. S., & Iqbal, M. (2014). Phytochemical investigation and free radical scavenging activities of essential oil, methanol extract and methanol fractions of nephrolepis biserrata. *International Journal of Pharmacy and Pharmaceutical Sciences*, *6*(9), 269-277. Retrieved fromhttps://innovareacademics.in/journals/index.php/ijpps/article/view/1512

Shewan, J. M., & Hodgkiss, W. (1954). A method for the rapid differentiation of certain nonpathogenic, asporogenous bacilli. *Nature*, *173*(4396), 208–209. https://doi.org/10.1038/173208b0

Shin, D., Byun, W. S., Moon, K., Kwon, Y., Bae, M., Um, S., Lee, S. K., & Oh, D. C. (2018). Coculture of Marine *Streptomyces* sp. With *Bacillus* sp. Produces a New Piperazic Acid-Bearing Cyclic Peptide. *Frontiers in chemistry*, *6*, 498. https://doi.org/10.3389/fchem.2018.00498

Sran, K., Bisht, B., Mayilraj, S., Roy C.A. (2019) Structural characterization and antioxidant potential of a novel anionic exopolysaccharide produced by marine Microbacterium aurantiacum FSW-25, *International Journal of Biological Macromolecule*, *131*,343–352 https://doi.org/10.1016/j.ijbiomac.2019.03.016

Stein T. (2005). Bacillus subtilis antibiotics: structures, syntheses and specific functions. *Molecular microbiology*, *56*(4), 845–857.https://doi.org/10.1111/j.1365-2958.2005.04587.x

Sujana, N., Ramanathan, S., Vimala, V., Muthuraman, M.S.,Pemaiah, B. (2012) Antitumour potential of *Passiflora incarnata*.L against ehrlich ascites carcinoma, *International Journal of Pharmacy and Pharmaceutical Sciences*, 4, 17-20.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular biology and evolution*, *10*(3), 512–526. https://doi.org/10.1093/oxfordjournals.molbev.a040023

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular biology and evolution*, *30*(12), 2725–2729. https://doi.org/10.1093/molbev/mst197

Wani, M. A., Sanjana, K., Kumar, D. M., & Lal, D. K. (2010). GC--MS analysis reveals production of 2--Phenylethanol from Aspergillus niger endophytic in rose. *Journal of basic microbiology*, *50*(1), 110–114. https://doi.org/10.1002/jobm.200900295

Xu, P., Ding, L., Wei, J., Li, Q., Gui, M., He, X., Su, D., He, S., Jin, H., (2020) A new aquatic pathogen inhibitor produced by the marine fungus *Aspergillus* sp. LS116, *Aquaculture*, 520, 734670.

Yadav, A.,Yadav, M., Kumar, S., Sharma, D., Yadav, J.P., (2018) *In vitro* Antioxidant Activities and GC-MS Analysis of Different Solvent Extracts of *Acacia nilotica* Leaves, Indian Journal of Pharmaceutical Sciences, 80(5),892-902.

Yogeswari, S., Ramalakshmi, S., Neelavathy, R., Muthumary, J., (2012) Identification and comparative studies of different volatile fractions from *Monochaetia kansensis* by GCMS. *Global Journal of Pharmacy and Pharmacology*, 6, 65–71.

Zhang, J., Li, B., Qin, Y., Karthik, L., Zhu, G., Hou, C., Jiang, L., Liu, M., Ye, X., Liu, M., Hsiang, T., Dai, H., Zhang, L., & Liu, X. (2020). A new abyssomicin polyketide with anti-influenza A virus activity from a marine-derived Verrucosispora sp. MS100137. *Applied microbiology and biotechnology*, *104*(4), 1533–1543.https://doi.org/10.1007/s00253-019-10217-2

Zhao, Y., Liu, D., Proksch, P., Zhou, D., & Lin, W. (2018). Truncateols O-V, further isoprenylated cyclohexanols from the sponge-associated fungus Truncatella angustata with antiviral activities. *Phytochemistry*, *155*, 61–68.https://doi.org/10.1016/j.phytochem.2018.07.017

***