DEVELOPMENT AND PHARMACOLOGICAL EVALUATION OF SMALL MOLECULAR BIOACTIVES FROM MARINE ALGAE ASSOCIATED HETEROTROPHIC BACTERIA

By

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THESIS

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2020

DECLARATION

I hereby declare that this thesis entitled "**Development and pharmacological evaluation of small molecular bioactives from marine algae associated heterotrophic bacteria"** is a bonefide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Cochin ANEETTA FRANCIS

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CERTIFICATE

Certified that this project report entitled "**Development and pharmacological evaluation of small molecular bioactives from marine algae heterotrophic bacteria**" is a record of project work done independently by Miss ANEETTA FRANCIS (2015-09-018) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him/her.

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DEDICATED TO ALL THE SCIENTIFIC MINDS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
^{O}C	Degree Celsius
µL	Microlitre
µM	Micromolar
3T3L-1	Fibroblast normal cell line
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid
BLAST	Basic Local Alignment Search Tool
bp	Basepair
cm	Centimeter
CAS	Chrome azurol sulphonate
DNA	Deoxyribo Nucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EtOH	Ethanol
g	Gram
h/hr	Hour
IC_{50}	Half Maximal Inhibitory Concentration of the population
IR	Infra-Red
LOX	Lipoxygenase
MCF-7	Human Breast Cancer cell line

MHA	Mueller-Hinton Agar
$MgCl_2$	Magnesium Chloride
MIC	Minimum Inhibitory Concentration
min	Minute
mL	millilitre
mm	millimeter
mM	millimolar
ng	nanogram
nm	nanometer
NMR	Nuclear Magnetic Resonance
No.	Number
PCR	Polymerase Chain Reaction
Rf	Retention Factor
RNase	Ribonuclease
ROS	Reactive Oxygen Species
Rpm	Revolution per minute
s	seconds
sp.	Species
TE	Tris-EDTA
TLC	Thin Layer Chromatography

UV	Ultra Violet
V	Volt
ZMA	Zobell Marine Agar

INTRODUCTION

1. INTRODUCTION

Accounting for two-thirds of the biosphere, marine ecosystems do play a major role as the hidden reservoir of potential flora and fauna with promising metabolic products (Harnedy *et al*., 2011). Marine natural products so far globalizing has paved its way from influencing nutritional supplements to cosmetics and on increased reach to pharmaceuticals (Guillerme *et al*., 2017; Donia *et al*., 2003). The association of marine eukaryota with its symbiont microorganisms has been the foundation for exploring the bioactive compounds that extends its applications to multiple fields of interest. Many marine organisms and their associated microorganisms have been experimented, and assessed resulting in the ominous products pertinent well enough in the natural product label. This combination has made it possible for the discovery of many compounds that were hidden from marine science so far.

Marine macroalgae or seaweeds are sessile multicellular photosynthetic eukaryotes with deficient specialized tissues as root system or vascular structures. Today these floras play an essential biodiversity role by harboring microbiomes with and within them (Egan *et al*., 2013). A great deal of studies and researches are in pace to deduce the structure, succession and dynamics of the microbial community associated with the marine algae and understand them better (Singh and Reddy 2014). The growing demand of new compounds from natural origin has led the limelight to the novel bioactive metabolites from symbiont bacteria, find their application in various industries. Among many marine inhabitants, seaweed harbors bacteria that impose strong antagonist activities when compared to other hosts of the ecosystem. (Chakraborty *et al.*, 2017).

Recently, studies involving algae, sponges and their associated bacteria unveiled that bioactive metabolites extracted from them have exceptional antibacterial and antioxidant activity, and related therapeutic applicability can be well exploited in drug discovery (Balakrishnan *et al.*, 2014; El-Moneam *et al.*, 2017). Due to the ability to occupy a variety of unique biological niches in an extremely challenging environment, marine heterotrophic microorganisms are considered an important source of novel metabolites deriving natural products (Gomez *et al*., 2010). With the growing necessity for novel drug discovery, these bacteria can represent a largely unexplored source for new antibacterial compounds (Penesyan *et al.*, 2009).

The bioactive compounds biosynthesized in response to the competition and predator deterrence, are the secondary metabolites produced by microbiome within (Gomez *et al.*, 2010). These metabolites require mainly three classes of enzymes for their synthesis, when put together are, polyketide synthases (*pks*), non-ribosomal peptide synthetases (*nrps*) and *pks*/*nrps* hybrids from chimeric genes giving chimeric products (Theobald *et al.*, 2019). The *nrps* and type I *pks* share to a large extent similar modular architecture and organized into modules containing multiple domains, allowing the repetitive incorporation of building blocks into larger resulting compounds (Aleti *et al.*, 2015). Polyketide classes of compounds are structurally divergent molecules with potential pharmacological properties, and were reported to occur in microorganisms and higher organisms (Winter *et al.*, 2016; Thilakan *et al.*, 2016). Interestingly, the studies stated that the polyketide compounds reported from macroalgae were in fact the compounds from their associated bacterial community (Chakraborty *et al.*, 2017). Bacillales, represent order in which the Bacillus genera of Firmicutes phyla comprise as a source for bioactive metabolites harboring biosynthetic gene clusters. Based on a whole genome mining study, 31 % of the *Firmicutes* in the study were estimated to harbor *nrps* and *pks* secondary metabolite gene clusters, and 70 % of those encoded *nrps* and 30 % hybrid *nrps*/*pks* or *pks* (Aleti *et al*., 2015).

Bacterial communities associated with seaweeds or macroalgae, are evolving as a diversifying and rich source of natural products of unparalleled chemical and structural diversity, that were unexplored since decades and shows to exhibit a broad range of potential biological activities (Goecke *et al*., 2010; Singh *et al*., 2015). Epiphytic representatives of the genera *Alteromonas, Bacillus, Pseudoalteromonas, Pseudomonas, Streptomyces* and *Vibrio* are recurrently reported as producers of antimicrobial molecules (Kanagasabhapathy *et al*., 2008; Wiese *et al*., 2009). Thus, macroalgae-associated microorganisms exemplify an attractive source of valuable bioactives with biotechnological and pharmaceutical applications.

Bioprospecting of marine microbes has recognized many astounding landmarks in pharmacology, drug designing, and therapeutics. Marine *Bacillus* are acknowledged to supply physically diverse secondary complexes like lipopeptides, non-ribosomal peptides, polypeptides, macrolactones, polyketides and coumarins emphasizing a good array of diverse pharmacophores, starting

from antimicrobial, antidiabetic and anticancer in nature, heavy metal detoxification, carotenoids production and even biocontrol agents and biopesticides (Cherian *et al*., 2019).

The present study aims to explore the bacterial communities associated with the marina algae and exploit them as a reservoir of pharmacologically potential compounds. The characteristic bacterial communities are analyzed by integrated identification methods and phylogenetically classified. Their potential to produce bioactive compounds is assessed using polymerase chain reaction (PCR) amplification of functional genes. These approaches are collectively undertaken in the present study 'Development and pharmacological evaluation of small molecular bioactives from marine algae associated heterotrophic bacteria'. The objectives of the proposed work summarize as:

(1) To evaluate the microbial diversity of bioactive producing marine algae-associated heterotrophic bacteria.

(2) To evaluate the pharmacological potential of the bacteria for anti-oxidant, anti-microbial properties.

(3) To identify the marine algae associated bacterial phyla by microbiological, biochemical and molecular characterization.

(4) To validate the pharmacological potential of the organic extract by target bioactive properties assessed *in vitro*.

The proposed research programme will help to identify the therapeutic potential of the marine algae associated heterotrophic bacteria from coastline of Peninsular India, and their possible pharmacological activities.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. MARINE ECOSYSTEM

The marine ecosystem covers almost two-thirds of the earth's total biosphere and harbor diverse biodiversity exclusive to marine niche. Marine ecosystem is a reservoir of industrial and pharmaceutically applicable compounds. The constant changes and adaptation processes had combating effect on entire ecosystem which evolved unique characteristics that enabled these habitats to allow the growth of organisms to adapt to these harsh conditions. The microorganisms play key role as producers of commercially important metabolites of biotechnological application. These metabolites find application in pharmaceutical, health, and as a solution to most encountered problem of antibiotic resistance in bacteria (Rao *et al*., 2017).

The antimicrobial activity has been extensively reported for extracts of varied groups of marine organisms (Blunt *et al.*, 2012). Even the antimicrobial properties reported from macroorganisms are a result of their symbiont microorganisms (Shnit-Orland and Kushmaro 2009). Furthermore, the extremophiles existing in the extreme conditions of the marina algae can be exploited because of their vast industrial and pharmaceutical application (Suriya *et al*., 2016). To get a better idea on evolutionary processes involved, deducing the metabolic responses of the marine species in both intraspecific and interspecific orientation and the mechanisms involved can possibly complete the picture on how marine ecosystem actually works (Stien 2020).

2.2. MARINE ALGAE AND THEIR SYMBIONT BACTERIA

The diversity of the marine ecosystem depends on the biodiversity present on the marine beds. Accounting for the nutritional supply for the overall marine ecosystem, marine algae pose a promising candidate for exploring their biological applications. Seaweeds or marine algae catch attention because of their collection of products exhibiting biological activities and biotechnological application (Chakraborty *et al*., 2017).

Macroalgae or seaweed and microalgae are the two types of algae found in marine ecosystem. Macroalgae includes three classes of algae commonly called green algae, red algae and brown algae. Microalgae is entirely distributed over the habitat and act as food for many marine

inhabitants (Garson 1989). Since 1900s, studies had been reported remarking the association of marine inhabitants and microorganisms. A broad range of beneficial or detrimental interactions between macroalgae and epi-and endosymbiotic bacteria have been found. Seaweeds provide themselves as suitable hosts for colonization by microorganisms. Every species of seaweed across the ecosystem harbor diversity of heterotrophic epiphytic bacteria and share a mutually beneficial relationship. The bacteria produce essential nutrients or metabolites that can confer protection against predators of hosts while in turn they provide a surface for their establishment. For instance, the heterotrophic bacteria utilize the oxygen and organic matter evolved by the algae and in turn mineralizes the organic matter, supplying the algae with nutrients and carbon dioxide.

The production of bioactive, anti-bacterial secondary metabolites is a result of dwelling competition between the existing microorganisms on the surface. Moreover, these dwelling competitions prevent the invading of free bacteria on the host surface due to the inhibitory spectrum of the bioactives produced. The constant balance between microbial communities is met by interspecific competition existing in the habitat. (Spoerner *et al*., 2012). The marine natural products are in fact chemical defenses that evolved into highly potent inhibitors prey, predators or competitors of the marine organisms that utilize them for survival or more simply said, self-defense mechanisms that are mutually beneficial to both symbiont and the host (Zhang *et al.,* 2005).

2.3. MARINE ALGAE ASSOCIATED BACTERIA

In a marine niche, the marine algae or the seaweed are the promising sources of microbial flora with bioactivity when compared to other eukaryotic hosts. Since the demand for the natural products are still hyper, the bioactive secondary metabolites are having increased demand for their administration in food and pharmaceutical industry.

Among the characterized metabolites, there are almost 22,000 known microbial secondary metabolites, of which 70% are produced by actinomycetes, 20% by fungi, 7% by *Bacillus* spp. and 1–2% by other bacteria (Subramani and Aalbersberg 2012).

Al-Zereini (2014) reported numerous compounds isolated from Red Sea bacteria with antimicrobial property along with cytotoxic activities. The sharing turned competition mechanisms among bacterial communities includes production of lysosomes, proteases antibiotics, bacteriocins, siderophores, and even the pH alteration through the assembly of

organic acids (Avendaño-Herrera *et al*., 2005). Additionally, the symbiont bacterial flora associated with the macroalgae produces and secretes diverse bioactive compounds with several antagonistic properties against Gram-negative and Gram-positive bacteria, antifouling and photosynthetic activity (Goecke *et al*., 2010). The marine macroalgal surface has been reported to invite micro and macroorganisms of adaptable nature. Subsequently, the symbiont microbial flora secretes chemical defenses that prevent any further colonization of microbes. Some microbes co-exist on same host surface that will mutually inhibit each other but will thrive on the host. Recent discoveries since last decade demonstrated that these symbiont microbial communities produce secondary metabolites of polyketide and non-ribosomal peptides chemistry. These bioactive compounds could be the reason to thrive over the hosts thus protecting them too. (Chakraborty *et al*., 2017).

2.4. TYPES OF SECONDARY METABOLITES PRODUCED BY MARINE BACTERIA

About 70% of them are secondary metabolites of marine bacteria, which give an inexhaustible source of diverse classes of non-ribosomally synthesized secondary metabolites, of which substances (lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, lipo amides and isocoumarins) are cyclic branched peptide compounds with unusual structures and structural templates of novel natural antibiotics (Andryukov *et al.,* 2019).

2.4.1. Cyclic Lipopeptides

Lipopeptides are a class of compounds with cyclic oligopeptides bound to fatty acids and that shows antibacterial activity against an array of pathogens. Iturins, fengycin, and surfactin are the three main categories of lipopeptides. Examples for characterized lipopeptides are tauromamid, halobacillin and methylhalobacillin derived from marine bacteria (Mondol *et al.,* 2013; Andryukov *et al.,* 2019).

2.4.2. Polyketides

Polyketides are medicinally important classes of secondary metabolites with medical and pharmacological application. The biosynthesis of these metabolites occurs with the involvement of multimodular megasynthases known as polyketide synthases (Andryukov *et al.,* 2019). The compounds from polyketide class of compounds include antibacterial (e.g.,

tetracycline), antifungal (e.g., amphotericin B), anticancer (e.g., doxorubicin), antiviral (e.g., balticolid), immune-suppressing (e.g., rapamycin), anti-cholesterol (e.g., lovastatin), and anti-inflammatory agents (e.g., flavonoids) (Rishdian *et al.*, 2019). PK are encoded by three types of gene clusters namely, *Type-I*, *Type-II* and *Type-III pks* enzymes. Macrolactones or collectively macrocyclic polyketides are produced by *Type-I pks* clusters and the type II PKS produces aromatic polyketide that includes compounds like anthracyclines, tetracyclines, tetracenomycins, and much more.

2.4.3. Non-ribosomal peptides

Nonribosomal peptides are a diverse class of secondary metabolites with contributions like toxins, siderophores, antibiotics, immunosuppressants or anticancer agents. They are encoded by multifunctional enzyme, non-ribosomal peptide synthases (NRPS) that translates into activated monomers of amino alkanoic acid building blocks. NRP are made structurally diverse by incorporating protein coding and non-protein coding amino acids into the backbone. These class of compounds are secreted by the microbes co-existing in association with the marine inhabitants. NRPs are produced by bacteria to combat the other microorganisms on the host surface for their colonization and for survival in harsh conditions which apparently prevents fatal predation to the hosts (Martínez-Núñez *et al*., 2016).

2.4.4. Isocoumarins

Isocoumarins are the secondary metabolites produced by microorganisms to higher eukaryotes. They are reported to possess strong antimicrobial activity against Gram-negative bacteria. Researchers have increased their interest to these compounds due to their anti-hypertensive ability, anticoagulant property, and contribute to antitumor activity by their anti-angiogenic. These compounds possess the common chromophore, 3,4-dihydro-8-hydroxyisocoumarin in their structures and lots of them are produced by the genus *Bacillus*.

2.4.5. Fatty acids

Fatty acids make up the hydrophobic part of the bacterial plasma membrane. The bacteria are synthesizing fatty acids by fatty acid synthase (FAS) pathway with chain length ranging from C12 to C19. The enzymes called fatty acid synthases (FAS) catalyses the production of fatty acids from acetyl-CoA and malonyl-CoA precursors.

2.4.6. Macrolactones

Macrolactones or lactone macrolides are the metabolites with potent antibacterial activity. They promote gastrointestinal motility in addition to their antagonistic property against microbes. The cyclization of polyketide chains assembled by PKS Type-I enzymes that perform repetitive decarboxylative condensations of carboxylic acids with an activated carboxylic acid starter unit leads to the formation of the macrolactins with macrolactone rings. Difficidin and oxidifficidin are the macrocyclic polyene lactones with strong antibacterial activity. A marine sediment derived *Bacillus* sp. Sc026 was characterized two macrolactins,7-O-succinyl macrolactin A and 7-O-succinyl macrolactin F, and another compound 7-O-malonyl macrolactin A from a B. *subtilis* was derived from marine algae evaluated for bioactivity (Mondol *et al.,* 2013).

2.5. MARINE *BACILLUS* SPECIES AND THE SECONDARY METABOLITES

Covering around 70 % of the earth's biosphere and inhabiting 80 % of the total life on earth, marine ecosystems does pose a reservoir of immense microbial diversity and new natural products of marine origin (Mondol *et al.,* 2013). Among the versatile microbial diversity, *Bacillus* covers the phylogenetically and morphologically diverse group of bacteria. They live in a pervasive marine environment and endure harsh conditions such as extreme temperature variations, pH variations, high pressure and salinity (Rampeletto 2010). Marine *Bacillus* competes with other microorganisms to race for nutrition and space. Due to this competition and dilute nature of ocean, bacteria produce strong bioactive compounds in order to survive the competition and micropredation (Stein 2020).

Marine *Bacillus* harbors unique classes of secondary metabolites like macrolactones, isocaumarins, lipopeptides, fatty acids and polypeptides having potential as anticancer, antibacterial, antimicrobial agents. Their application as a biocontrol agent can be used against phytopathogens also as they grow rapidly in liquid media and can form resistant spores. *Bacillus* species can proliferate on every alga, except *Egregia menziesii*. Antibiotic production has been reported that *Bacillus brevis, B. cereus, B. circulans, B. laterosporus, B. licheniformis, B. polymyxa, B. pumilus, and B. subtilis* making them an exploitable source of natural products (Madigan *et al*., 2005). Genus *Bacillus* is found on the surfaces of various hosts in the marine niche and is most predominantly present on red and brown algae

(Kanagasabhapathy *et al*., 2008). Many antimicrobial compositions have been isolated from bacterial-algal interactions. Karthick and Mohanraj (2018) reported a potential *Bacillus* sp. that showed notable activity against most of the tested pathogens, including toxin producing pathogens like *Shigella boydii*, Enterotoxigenic *E. coli*, Shigatoxin producing *E. coli*, *E. coli*, and *A. hydrophila* etc. Similarly, from the brown algae *Sargassum swartzii* two isolates were derived to name *Bacillus* sp. SG107 and *B. sphaericus* SG115 that showed moderate to less activity against few pathogens.

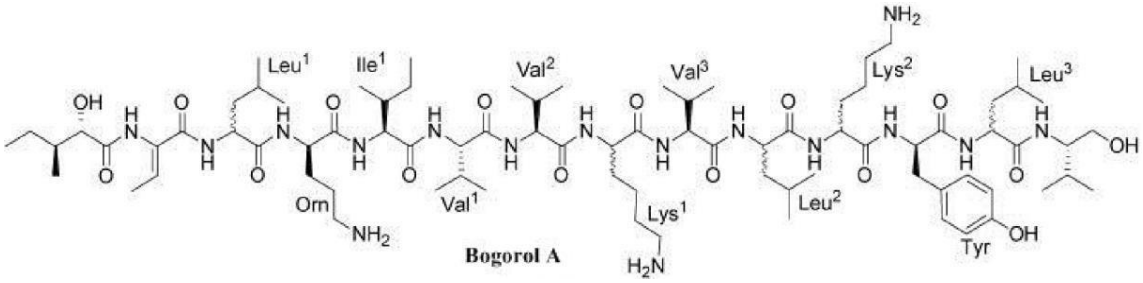
Another class of secondary metabolites is made of hybrid PKS/NRPS genes that produce different compounds in either orientation. In case of PKS-NRPS hybrid products, they have a polyketide backbone incorporated with amino acids. The NRPS-PKS products possess structure that have a peptidyl chain with ketone group. Three iturin classes of compounds, mycosubtilin, iturin A and bacillomycin D are reported from various strains of *Bacillus* spp. (Fickers 2012).

2.5.1. Antimicrobial agents

Cyclic lipopeptides are the class of compounds mainly produced by marine Bacillus group. CLP are divided into three families: iturins, fengycins, and surfactins. Since the last two decades, the most studied group of bacteria that produce anti-microbial products hail from the phyla *Firmicutes* in which the genera *Bacillus* predominates (Stincone and Brandelli 2020).

Studies carried out in some seaweeds displayed that they share alike defense mechanisms and impart inhibition for the growth of Gram negative *Proteus mirabilis* and *Klebsiella pneumoniae* (Villarreal-Gómez *et al*., 2010). Most of the metabolic responses by the higher eukayotes are infat, a result of the metabolic products of the symbiont microorganisms. It showed that they were excellent candidates for naturally occurring bioactive products. Lemos *et al*. (1985) demonstrated that 17 % of 224 isolates associated with five species of green and brown algae displayed antibacterial activity. Similarly, Penesyan *et al*. (2009) isolated 325 bacterial isolates from *Delisea pulchra* and *U. australis* and 12 % of them showed antibacterial activities. Another study by Kanagasabhapathy *et al*. (2008) reported that 33 % of epiphytic bacterial strains exhibited antibiotic activities that were associated with nine species of red alga in the study.

A novel protein was obtained from *B. licheniformis* associated with *Fucus serratus* with antibacterial activity which showed inhibition against MRSA, VRE, and *Listeria monocytogenes* (Jamal *et al*., 2006).



Bogorol A

Bogorol A **(1)**, a novel peptide antibiotic obtained from marine *Bacillus* sp. of tropical reef showed good activity against MRSA with a MIC of 2 µg/mL, VRE with MIC 10 µg/mL and moderate activity against *E. coli* (MIC 35 µg/mL. Prieto *et al.* (2012) suggested that seaweed-associated *Bacillus* spp. could be a source for novel bacteriocin when he discovered a bacteriocin named lichenicidin member of lentibiotics group of compounds from seaweeds-associated *B. licheniformis*. Consequently, another 8 kDa weighing bacteriocin from seaweed-associated *Staphylococcus haemolyticus* MSM was resulted to exhibit strong antibacterial activity against human pathogenic bacteria was partially characterized (Suresh *et al*., 2014).

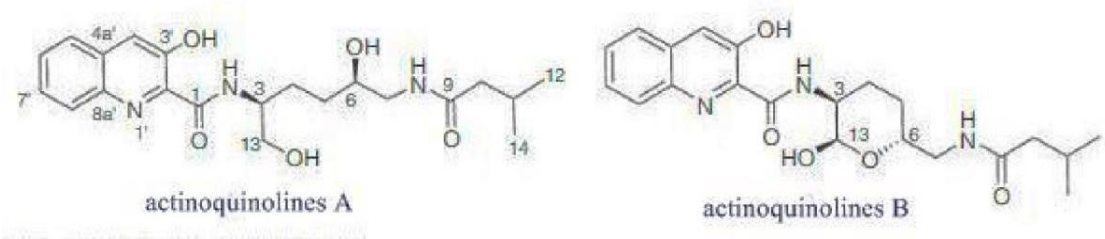
Similarly, three gene clusters corresponding to difficidin, macrolactin and bacillaene were reportedly found in the genome of B. *amyloliquefaciens.* Difficidin exhibits antibacterial activity by inhibiting protein synthesis in pathogens. Bacillaene displays antibacterial activity against human pathogens *Serratia marcescens, Klebsiella pneumoniae* and *Staphylococcus aureus* by inhibition of prokaryotic protein synthesis (Fickers 2012).

Thilakan *et al*. (2016) reported the extraction of antimicrobial furanoterpenoids from marine algae associated *Bacillus subtilis* MTCC 10403 exhibiting strong antagonist activity against *Vibrio spp.* of class of human pathogens. Two new antibacterial O-heterocyclic compounds belonging to polyketide origin with pyranyl benzoate analogs showcased activity against pathogenic bacteria. The characteristic compound was contributed by *B. subtilis* MTCC 10407 associated with the marine algae (Chakraborty *et al*., 2017).

2.5.2. Anti-inflammatory agents

Anti-inflammatory agents block the instigation and development of inflammation-associated diseases by suppressing the pathway of the inflammatory mediator production, which results in a decrease of inflammatory response. COX and LOX enzymes have substantial roles to play in the direction of inflammatory responses. The LOX enzyme which produces leukotrienes is important in the pathophysiology of inflammatory diseases. Kurian *et al.* (2015) reported that at lower concentration, marine *Bacillus spp.* BTCZ31 melanin inhibits LOX activity. Melanin with an IC_{50} concentration of 104.34 µg/mL inhibits COX enzyme, while LOX enzyme was inhibited with an IC_{50} value of 10.5µg/mL.

The epiphytic bacteria, *B. amyloliquefaciens* MTCC 12716 and *S. algae* associated with marine macroalgae showed strong anti-inflammatory activity. The crude extract of *B. amyloliquefaciens* displayed inhibitory activity against COX-2 and 5-LOX enzymes with IC_{90} 29 and 6.06 µg/mL, and *S. algae* displayed activity with IC_{90} 44 and 7.73 µg/mL, respectively. (Kizhakkekalam and Chakraborty 2019).



The bacteria *Streptomyces* spp. found in marine sediment produced quinolone alkaloids actinoquinolines A (3) and B (4) that were able to inhibit COX-1 and COX-2 activity *in vitro* (Souza *et al.*, 2020).

2.5.3. Antihyperglycemic agents

Even though the pathophysiology and the complex regulatory networks involved in the initiation of diabetes is still remaining undercover, evidence reports that oxidative stress along with inflammation processes are the driving forces for the initiation of the hyperglycemia or simply put, diabetes. Consequently, the characterization of anti-diabetic compounds includes tests to evaluate their anti-inflammation and antioxidant properties. The screening includes tests such as the activation of free radical detoxification enzymes/proteins or radical scavenging activity and inhibition of inflammatory mediators (Lauritano and Ianora 2016). The heterotrophic bacteria, *B. amyloliquefaciens* MTCC 12716 (IC_{90} 84 µg/mL) and *S. algae* (IC_{90} > 600 µg/mL) showed significantly greater inhibitory activity against *α*-glucosidase than acarbose (IC_{90} 645 µg/mL), the drug standard (Kizhakkekalam and Chakraborty 2019).

Streptomyces corchorusii subsp. *Rhodomarinus*, a marine derived actinomycete showed promising inhibitory activity against α-amylase inhibition. Pyrostatins A and B are the two novel compounds with strong anti- *N*-acetyl-glucosaminidase activity extracted from *Streptomyces* sp. isolated from Otsuchi Bay in Iwate (Lauritano and Ianora 2016).

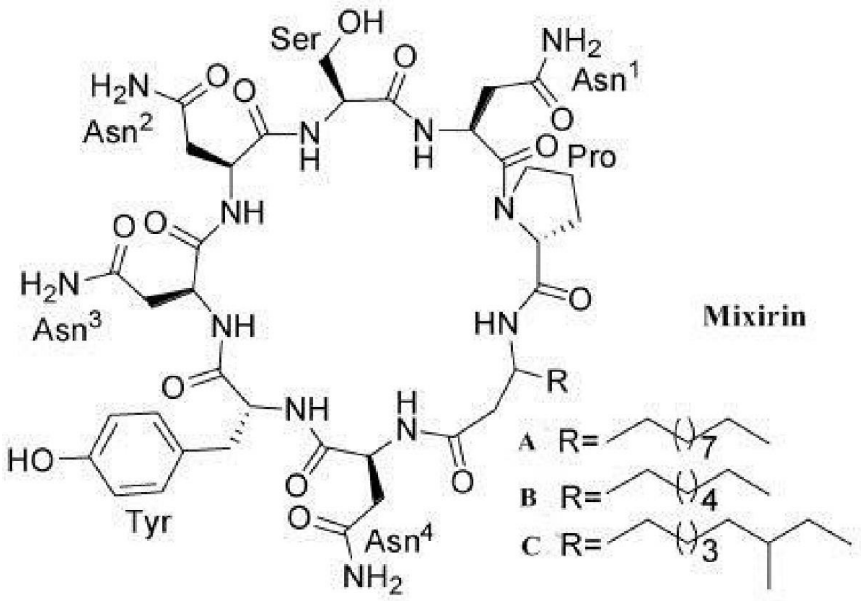
2.5.4. Anticancer agents

Halobacillin and methylhalobacillin are two cyclic acylpeptide isolated from *Bacillus* sp obtained from deep-sea sediment core. These compounds have reported to have anti-cancer properties against Human HCT-116 cancer cells with a IC_{50} concentration of 0.98 µg/mL (Andryukov *et al.*, 2019). In studies involving symbiont bacteria, it was found that bacteria of the family *Firmicutes, Proteobacteria* and *Actinobacteria* associated to the seaweed surface produced antitumor metabolites that inhibited the growth of HCT-116 colorectal cancer cells (Villarreal-Gómez *et al*., 2010).

Marine *Pseudomonas*-derived bioactive substances are diverse and include pyrroles, pseudopeptides, pyrrolidinedione, phloroglucinol, phenazine, benzaldehyde, quinoline, quinolone, phenanthrene, phthalate, andrimid, moiramides, zafrin and bushrin. Some of these bioactive compounds are antimicrobial agents, whereas dibutyl phthalate and di-(2-ethylhexyl) phthalate have been reported to be cathepsin B inhibitors. Discodermolide, bryostatins, sarcodictyin,

and eleutherobin are among the most effective anticancer drugs produced mainly by marine bacteria.

Mixirins A, B, and C that showed cytotoxicity activity against human colon carcinoma cell line HCT-116 was isolated from *Bacillus sp.,* from marine sediments (Zhang *et al*., 2004). Pyrroloformamide, produced by the actinobacteria *Streptomyces sp.*, a symbiont with the ascidian *Eudistoma vannamei* exhibited bioactivity with an IC_{50} value of 1.67 µM that inhibited cell division in the prostate cancer cell line PC3M (Abreu *et al*., 2014). Another compound Cromomycin A2, extracted from *Streptomyces sp.* stimulated autophagy with a concentration of IC_{50} of 16.7 nM in the metastatic melanoma cell line MALME-3M (Guimaraes *et al*., 2014).



2.6. FUNCTIONAL GENES TO FOSTER SECONDARY METABOLITE PRODUCTION

Microorganisms have found various methods to ensure their survival and adaptations in the marine niche among the field of competition. In order to win the survival, they produce some defense modules, collectively called secondary metabolites. These can be antimicrobial in nature, antifouling in nature, and even nourishment for the symbiont host.

The classes of secondary metabolites are a result of complex biochemistry and genetic adaptation to the evolved self-defense and aspects of microbial life. PKS and NRPS are the most

studied complex protein classes from which exclusive metabolites have been created for commercial and pharmaceutical application (Hutchinson 2003).

Polyketides (PKs) are class of secondary metabolites expanded from bacterial, fungal, and plants sources having vital biological potential and find their application as immunosuppressants and could be used as antibiotics, antifungals, and anticancer drugs (Hill *et al*., 2010). Polyketides are a large, complex and structurally diverse bioactive metabolic product synthesized exclusively by a group of multifunctional enzymes called polyketide synthases that have ketosynthase (KS) domain exhibited in each module and conservative in all domains. Thus, KS domains can be compared to retrieve the evolutionary studies (Zhang *et al*., 2009).

Another class of compounds that shows similar functionalities but are structurally diverse collectively called nonribosomal peptides (NRP). NRP are extensive family of compounds produced independent of ribosomal machinery of which the metabolites are distributed as immunosuppressants, toxins, anticancer agents, siderophores, pigments, or antibiotics (Martínez-Núñez *et al.,* 2016). The enzyme NRPS encode for the NRP class of compounds via a thiotemplate mechanism with three domains, adenylation (A) domain, peptidyl carrier protein (PCP) or thiolation (T) domain, and condensation (C) domain for the synthesis of NRPs (Martínez-Núñez *et al.,* 2016).

Genome mining along with synthetic biology has been evolved lately in designing genetic circuits and/or the redesign of existing biological systems are mainly used to detect novel natural products in bacteria and fungi with association of their potential metabolic function. This approach is probably because of operon organization of their synthesis genes (Albarano *et al*., 2020). Discovery of new nonribosomal peptides can be done by genome mining tools targeting the NRPS genes. The investigation of sequenced genomic DNA based on bioinformatics analysis has been extensively applied to identify a specific nonribosomal peptide gene cluster and its corresponding natural product (Wei *et al*., 2018).

Additionally, finding new biosynthetic gene clusters (BGCs) remains the central role of genome mining process. Indeed, the BGCs encode the enzymes, PKS and NRPS which are the two most essential biosynthetic clusters for translation of natural products. The association of genome mining with bioprospecting techniques could lead to the isolation of novel molecules of pharmacological interest (Fickers 2012).

2.7. NEED FOR BIOACTIVE COMPOUNDS

The need for naturally occurring bioactive compounds was always on pace. The demand increases with increasing population. With the experimented and established results of their pharmacological and industrial application, natural products from marine origin have started to be acknowledged globally. Along with this, main fiasco is the emergence of resistant bacteria that are proving deadly to human and aquatic life. With the aim of combatting multidrug resistant bacterial pathogens, new antibiotics are instantly required to prevent deadly human infections. For this line of study, much effort has been implemented to find novel compounds with bioactivity from marine organisms such as molluscs, sponges and soft corals. Another hurdle remains the inability to culture these microorganisms at laboratory level, and their presence only being validated by using molecular, biological and microscopic techniques. The reports for the need of bioactive compounds from earlier studies or publications indicate that the need was high since then and still counting.

In 1985, Lemos *et al*. demonstrated that seaweed associated bacteria produced antibacterial compounds. Further, it was determined that bacterial communities in fact secrete antibacterial secondary metabolites. Almost 25 % of the bacteria associated with seaweeds and marine invertebrates are capable of producing metabolites that have strong antagonist activity against methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE; Mearns-Spragg *et al*., 1997). The newly evolved antibiotic resistant bacteria, like Methicillin Resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecium* resistant to Vancomycin prove to be the reason behind most of fatal infection confronted in the primary line of healthcare (Soria-Mercado *et al* 2011). Their evolved mechanisms can cancel out the antimicrobial activity of the currently available drugs and cause hurdles to combat these bacteria. The decreased efficiency and continuous application of a drug can eventually lead to the acquiring of the resistance to that drug. Therefore, new drugs with novel bioactivity need to be discovered to fight these resistant microorganisms. Recently, β-lactamase, also named NDM-1, gives resistance to the wide spectrum of the β-lactams antibiotics. The genes that are linked to this enzyme are easily transferred between common bacteria and no treatment line are yet discovered (Soria-Mercado *et al* 2011).

With this foundation, we envisage to add another bioactive isolate that can stand up to minimize the increasing concerns of the day. So this study aims to contribute bioactive producing bacterial isolates to the repository of the potential pharmacophore producing bacteria with promising antibacterial properties and other therapeutic potential associated within them. As per the literature reports, the bacteria associated with the surface of the marine macroorganisms is capable of producing bioactive compounds, we envisage to isolate and characterize them for better exploitation and application.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Location

The study entitled 'Identification of small molecular bioactives from marine-algae associated heterotrophic bacteria and evaluation of their pharmacological potential was carried out at the Marine Natural Product Laboratory in Marine Biotechnology Division of ICAR-CMFRI, Kochi starting from October 1 2019 to 30 June 2020. In this chapter, the materials and methods employed in the study are explained in detail.

3.2. Sample collection

The marine macroalgae belonging to the classes of *Rhodophyceae,* and *Phaeophyceae* were collected from the coastal area of Mandapam, Gulf of Mannar region and Vizhinjam harbor, in the Arabian Sea, at the Southern Coast of Peninsular India. The macroalgal samples were transferred to a sterile polythene bag with seawater and kept in the dark at 4 °C until further processing in the laboratory. Symbiotic heterotrophic bacteria associated with the marine macroalga *Sargassum wighti, Gracilaria edulis, Turbinaria ornata, Padina pavonica, Sargassum tenerrium, Sargassum marginatum, Laurencia papillosa* were isolated and cultured by following previous reports of literature (Quevrain *et al*., 2014; Thilakan *et al*., 2016).

3.3. Isolation of marine macroalgae-associated heterotrophs

The algae specimen samples (1 g) were thoroughly washed in sterile distilled water to remove dirt and loosely attached microbes. Then, they were suspended in sterile seawater (10 mL) and homogenized using a pestle and mortar in a laminar airflow hood under an aseptic condition. Apparently, 1 mL of the suspension was added to sterile distilled water (9 mL), and different dilutions were prepared and plated on the Zobell Marine Agar (ZMA) and Nutrient agar (NA), supplemented with sodium chloride (NaCl, 1% w/v). The plates were incubated in the dark at 30 °C for 7 days. The pure colonies were obtained by subsequent isolation and purification steps on NA supplemented with NaCl (1% w/v) (Wiese *et al*., 2009; Chakraborty *et al*., 2014). Streak plate method was used to isolate a pure culture.

The microbes were transferred from one growth medium to another to keep them alive and for their proper growth. A total of 31 well grown and morphologically distinct bacterial cultures from the samples were subcultured into sterile NA plates. The bacterial cultures were further quadrant streaked to obtain pure cultures and single colonies were picked and streaked in NA slants. The isolates were incubated at 34 °C and further stored at 20 °C in the BOD incubator (Labline). The pure cultures were inoculated into NA (with 1% NaCl) by stab method and glycerol was added onto it. These glycerol stab stocks were stored in 20 °C in the BOD incubator (Labline) for long term storage of the cultures.

3.5. Preliminary antibacterial screening by spot-over-lawn assay

Preliminary screening of the heterotrophic isolates was performed by spot-over-lawn assay (Chakraborty *et al*., 2014). A lawn of the pathogenic bacteria was grown on Mueller Hinton agar (MHA) (HiMedia) plates, over which the isolates were spotted using sterile swab or toothpick. The test pathogenic organisms were *Vibrio parahaemolyticus* (MTCC 451), *Aeromonas salmonicida* (ATCC 27013), *Yersinia enterocolitica* (MTCC 859), *Escherichia coli* (MTCC 443), *Streptococcus pyogenes* (MTCC 1924), *Edwardsiella tarda* (MTCC 2400), *Aeromonas caviae* (ATCC 15468), and multi drug resistant *Staphylococcus aureus* (MRSA). The plates were incubated at 34 °C and the antibacterial activities were recorded according to the inhibition zone developed on the plates after a period of 24, 48 and 72-h incubation. The diameter of the zone of inhibition was measured using antibiotic zone scale (HiMedia).

3.6. Biochemical identification of the selected isolates

The isolates were selected from preliminary anti-microbial activity. The bacteria that showed inhibition zone against most of the pathogens were only selected for further biochemical and molecular characterization. Hence, bacteria with strong antimicrobial properties were identified using biochemical methods. The colony morphology was analyzed on agar plates and Gram staining was performed. The strains were identified by biochemical and physiological tests as described in the *Bergey's Manual of Determinative Bacteriology* (Krieg and Holt 1984). Conventional tests, such as motility, starch hydrolysis, gelatin hydrolysis, growth at various temperatures and NaCl concentrations, HiMedia HiBacillus Identification kit including tests for

malonate, voges-proskauer, citrate, ONPG, nitrate reduction, catalase, arginine, sucrose, mannitol, glucose, arabinose, and trehalose were used.

3.6.1. Gram staining

Gram staining was done by conventional methods on glass slides. A drop of distilled water was taken onto the center of the slides and a loop full of organisms was mixed and smeared on the glass slide. The smear was air dried and heat fixed for further staining procedures. The primary stain Crystal violet was added onto the smear and allowed to stand for 1 minute and washed off with distilled water. The smear-stain complex was fixed by mordant, Gram's iodine with an incubation of 1 minute and washed off. The crystal violet was decolorized from the smear using decolorizer (ethanol/ acetone) by pouring them onto it. It was immediately washed off using distilled water and a counterstain was administered. Saffranin gave a characteristic pink color to the cells after the incubation of 1 minute. The slide was air dried and viewed under a compound microscope. Here, the slide was viewed under 10X, 40X, 100X using an inverted microscope (LEICA). Cedar wood oil was added onto the smear and viewed under 100X (Oil immersion). The bacteria which showed purple color were Gram-positive and which showed pink color were Gram-negative.

3.6.2. Motility test by Motility Indole Lysine (MIL) medium

A combination of motility, lysine decarboxylation, lysine deamination, and indole production tests was done by MIL medium. The MIL medium is autoclaved and solidified. The culture is then stabbed onto the center of the medium and incubated at 37 ℃ for 24 hours. The presence of growth along and away from the stabline denotes motility. If the medium turns purple shows positive lysine decarboxylase and a red-brown color in the top of the medium shows positive lysine deamination reactions. On addition of Kovac's reagent, a presence of red ring at interface of medium and reagent denotes indole production.

3.6.3. HiBacillus Identification Kit

A pure colony was picked from test organisms and inoculated into 5 mL Brain heart infusion broth and incubated at 35-37 ℃ for 6 to 8 hours until the inoculum turns turbid. The kit was aseptically peeled off and 50 µL of inoculum is added into each well by surface inoculation method. The kit is then incubated at 34 ℃ for 24- 48 hours.

3.6.3.1. Malonate

This tests the malonate utilization by color change of medium from bluish green to dark blue.

3.6.3.2. Voges-Proskuer's

This test detects the acetoin production by color change of medium from light yellow to pinkish red on addition of Baritt's reagent.

3.6.3.3. Citrate

This test detects the citrate utilization of the inoculum by changing the green color of the medium to blue color.

3.6.3.4. ONPG

This detects the presence of beta-glucosidase with color change from colorless to yellow of the medium.

3.6.3.5. Nitrate reduction

This detects the nitrate reduction capacity of the inoculum by color change from colorless to pinkish red on addition of sulphanilic acid and N, N-dimethyl-1-naphylamine.

3.6.3.6. Catalase

It detects the catalase activity by the effervescence coming out from the loop of the organism when immersed in hydrogen peroxide.

3.6.3.7. Arginine

It detects arginine utilization by changing the color of the medium from olive green to purple.

3.6.3.8. Carbohydrate tests

It detects the carbohydrate utilization by changing the color of the medium from pinkish red to yellow.

3.7. Preliminary screening for antioxidant activity

The ability to scavenge DPPH is assayed *in vitro* by filter paper method. The selected isolates were inoculated onto sterile NA plates and incubated for 24 h at 37 °C. On the other day, 90 mm Whatman filter paper was placed aseptically onto grown cultures and incubated for another 24 h at 37 °C. The filter paper was taken and placed in a sterile petri plate. To assess the antioxidant property, 0.2 mM solution of DPPH was sprayed onto the filter paper. The portions showing clear zones were measured and expressed in mm.

3.8. Genomic DNA isolation

The selected bacterial cultures were grown in nutrient broth and the genomic DNA was extracted using GenElute^{TM} Bacterial Genomic DNA kit (Sigma- Aldrich). The bacterial cultures were grown in nutrient broth for 18 h and the cells were pelleted out by centrifugation at 12,000g x g for 2 min. A 200 µL of lysozyme solution was added to Gram positive bacteria with 30 min incubation at 37^{O} C and 180 µL of lysis solution T contained in the kit to Gram negative bacteria. RNase A (20 µL) and Proteinase K (20 µL) were added to remove RNA and protein contamination. The mixture was further incubated at 55 ^{O} C for 10 min. The DNA was isolated according to manufacturer's protocol. The genomic DNA of the selected bacteria were dissolved in the Tris-EDTA (TE) buffer and kept in -20 ^{O} C for long term storage.

3.9. Agarose Gel Electrophoresis

Agarose gel electrophoresis allows the separation of DNA fragments according to their size. The ethidium bromide helps in visualizing the DNA as bands under UV illumination. Here, 0.2 g of agarose was added onto a 25 mL Tris-Boric acid-EDTA (TBE) buffer and heated in the microwave until completely dissolved. On reaching the appropriate temperature, ethidium bromide is added, then mixed and poured onto a casting tray. The comb is inserted and allowed to solidify. Further the agarose gel is transferred to the electrophoresis tank and filled with TBE buffer. The samples are loaded on to wells and the electrophoresis is run at 70 V and 35 mA until the tracking dye runs 3/4th of the gel. The gel is then visualized under UV illumination to detect the DNA bands and is documented in the Gel documentation system (Biorad, USA). The DNA samples were further stored in -20°C.

3.10. Quality check using nanodrop spectrophotometer

The quality and quantity of the DNA samples were assessed using a nanodrop spectrophotometer (Eppendorf, Biospectrometer). The equipment was calibrated using TE buffer to blank. Absorbance was assessed at 260/280 nm and the concentration of the sample (ng/µL) were observed.

3.11. 16S rRNA gene amplification

The isolates were identified by molecular characterization by 16S rRNA gene amplification. The molecular characterization of the cultured bacterial strains was performed by 16S rRNA gene sequencing assisted with BLAST similarity search. PCR was performed in a total volume of 25 µL containing 2X PCR master mix (Takara), 1 µL of each primer (Sigma), 1 ng of DNA. The following cycling conditions were used: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72°C for 2 min, with a final extension at 72 °C for 5 min in the thermo cycler (Biorad, USA). The molecular sizes of the amplified fragments were assessed with a 1 kb ladder on a 1.5% (*m*/*v*) agarose gel in 1X TBE buffer. The amplicons were sequenced for molecular identification.

3.12. 16S rRNA-based phylogeny and phylogenetic analysis

The gene sequence retrieved from the amplicons was subjected to blastn search to compare with the existing sequences and the sequence data were deposited in GenBank for accession number. The sequences were aligned in the BioEdit software with CLUSTALW application against the reference sequences retrieved from NCBI blast search program. The aligned data set was used as input for the phylogenetic analysis program. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model and phylogenetic analyses were carried out in MEGAX (Tamura and Nei 1993).

3.13. Molecular identification of functional genes

Identification of functional *pks/nrps* genes was carried out by PCR amplification of the specific genes using degenerate primers. PCR was carried out in a reaction volume of 25 µL with 12.5µL 2X PCR Master mix (TAKARA), 1ng DNA, 1µL each of forward and reverse primer and final volume made up using sterile water. PCR was carried out at initial denaturation at 94^0 C for 5 min, 35 cycles of denaturation at 95^0 C for 1 min, followed by annealing at 45^0 C for 1 min for PKS and at 55^0 C for NRPS, extension at 72^0 C for 1 min, and the final extension at 72^0 C for 5min. Agarose gel (1.5%) was used to detect the amplified products as fragments, by agarose gel electrophoresis, and bands of 700 bp and 1000–1400 bp were considered as products of *pks-I and nrps* genes, respectively. The products were sequenced and the sequences were submitted for the accession numbers in NCBI GenBank.

Table 3.1. Polymerase chain reaction (PCR)† primers used for 16S rRNA and *pks*, *nrps* gene amplification‡

Primer	Target	Sequence (5′-3′)	References
16S1	*16S rRNA*	GAGTTTGATCCTGGCTCA	Xia *et al*. 2015
16S2	*16S rRNA*	ACGGCTACCTTGTTACGACTT	Xia *et al*. 2015
GCF	*pks*	GCSATGGAYCCSCARCARCGSVT	Schirmer *et al*. 2005
GCR	*pks*	GTSCCSGTSCRTGSSCYTCSAC	Schirmer *et al*. 2005
GBF	*pks*	RTRGAYCCNCAGCAICG	Zhang *et al*. 2009
GBR	*pks*	VGTNCCNGTGCCRTG	Zhang *et al*. 2009
KS11F	*pks*	GCIATGGAYCCICARCARMGIVT	Schirmer *et al*. 2005
KS12R	*pks*	GTICCIGTICCRTGISCYTCIAC	Schirmer *et al*. 2005
KSDPQQF	*pks*	MGNGARGARGCNNWNSMNATGGAYCCN CARCANMG	Zang *et al*. 2009
KSHSGDR	*pks*	GGRTCNCCNARNSWNGTNCCNGTNCCRTG	Zang *et al*. 2009
MTF	*nrps*	GCNGGYGGYGCNTAYGTNCC	Zhao *et al*. 2008
MTR	*nrps*	CCNCGDATYTTNACYTG	Zhao *et al*. 2008

†The PCR reaction was carried out in a reaction mixture of total 25 µL that consists of 12.5 µL of 2X PCR master mix (TAKARA), 1 µL each of forward and reverse primers (Sigma), 1 µL of the DNA, and final volume made up by sterile water.

‡Different sets of oligonucleotide primers were used to amplify the partial region specific for *pks* and *nrps* genes as described. The resulting amplicons of size 700 bp correspond to *pks-I* specific genes while 1000 bp-1500 bp amplicons gave + results for *nrps* genes.

3.14. Antibiotic susceptibility

Antibiotic sensitivity of the isolate selected by spot-over-lawn assay and *pks-I* screening was determined through octadiscs (HiMedia), commercially available with of eight antibiotics

infused— erythromycin, gentamicin, cephalothin, clindamycin, trimoxazole, vancomycin, ofloxacin, and Penicillin-G. The disc was placed on the center of the MHA plate over the bacterial lawn, following the guidelines by CLSI (Kizhakkekalam *et al*., 2020).

3.15. Siderophore detection assay

The bacterial strain was checked for siderophore-production by universal chrome azurol sulfonate (CAS) assay (Schwyn and Neilands 1987). Briefly, 0.06g of Chrome Azurol S (CAS) was dissolved in 50 mL of distilled water and added to 9 mL of 1 mM Ferric ferric chloride ($FeCl_3 \bullet 6H_2O$) prepared in 10 mL of 10 mM HCl. This solution was added to hexadecyl trimethyl ammonium bromide (HDTMA) solution (0.073 g in 40 mL distilled water) under stirring. The blue color CAS-HDTMA solution was sterilized and stored. Following, a minimal media was prepared consisting of 1.5 g KH_2PO_4, 2.5 g NaCl, and 5 g NH_4Cl in 50 mL distilled water. The CAS agar was prepared by mixing the minimal media and PIPES buffer. The pH of the solution was made up to 6.8 by 10 % NaOH solution. A 1.5 g of agar was added to the solution and sterilized. Further, 30 mL of Casamino acid along with 10 mL of 20 % sterile glucose solution was added onto the media and solidified. The bacterial strain was spotted on the CAS agar plate. After inoculation, the plates were incubated at 37 ℃ for 5–7 days and observed for the formation of a yellow-orange zone around the bacterial colony (Louden *et al*., 2011).

3.16. Bacterial growth curve

The screened and molecular characterized candidate *Bacillus velezensis* from the 3 isolates was selected for the extraction of secondary metabolites. The specified candidate showed promising anti-microbial activity with positive screening for functional *pks/nrps* genes. Thus, the culture was inoculated onto sterile nutrient broth supplemented with 1% NaCl and incubated at 37° C for 16-18 h. About 5 mL of inoculum was added onto 95 mL sterile nutrient broth and absorbance was measured in UV-VIS spectrophotometer (Varian, Cary) at 660 nm for 76 h.

3.17. Preparation of crude extracts from *Bacillus velezensis* MBTDLP1 and *in vitro* bioassays

The extracellular metabolites of the selected bacteria *B. velezensis* MBTDLP1 were extracted

with a suitable organic solvent. The bacteria were surface cultured over nutrient agar and incubated at 34 °C for 72 h. The culture on the surface was removed by scrapping, and the agar with the extracellular metabolites was cut into small pieces which were exhaustively extracted by refluxing with organic solvent ethyl acetate on a heating mantle for 6 days. The organic extract was passed through sodium sulphate to remove any water content. The metabolites were concentrated on a rotary vacuum evaporator (Heidolph, Schwabach, Germany). The organic extract was dried, passed through nitrogen and sealed using paraffin wax and was stored in 4 °C until further analyses. The organic extract of the selected bacteria was assayed *in vitro* for their pharmacological properties including anti-bacterial, anti-oxidant, anti-inflammatory, anti- diabetic and anti-cancer property, respectively.

3.18. Ultrasonication for bacterial cell lysis

Ultrasonication was performed to retrieve the intracellular metabolites of the isolate. The scraped out bacterial cells from the nutrient agar surface were transferred to 25 mL ethyl acetate. The cells were lysed at 40% amplitude and the timer was set for 10 second on and 5 second off cycle for a total of 15 minutes in ultrasound sonicator (Cole-Parmer). The intracellular metabolites dispersed into the solvent are filtered through sodium sulphate to remove water content if any. Fresh solvent is added and metabolites again extracted from cells using ethyl acetate on a temperature controlled heating mantle. The extract is passed through sodium sulphate and is concentrated *in vacuo* in a rotary vacuum evaporator.

3.19. Thin layer chromatographic documentation and visualization

Thin layer chromatography was carried out to detect the compounds in the organic extract. The extract was spotted in TLC plates coated with silica with a capillary tube. The plates were then run in TLC chambers against several solvent systems in different concentrations for better mobilization of compounds in the TLC plates. Finally, 100% ethyl acetate was selected as the ideal solvent system for chromatographic run for the specific extract. The plates were then dried and viewed under UV light in 254 nm and 365 nm. Compounds with extended conjugation, and aromatic rings which are UV-active, can be visualized under UV illumination. The documentation of the same was done using ELITE-MINI LUMINOUS, AE-04 (Aetron, Mumbai, India). The retention factor was calculated for each compound. The visualization

reagents were used to stain the plates and detect different classes of compounds. The plate was sprayed with 10 mL 5% or 10% Ethanolic potassium hydroxide (Bornträger reaction) and was evaluated in vis. or in UV-365 nm, with or without warming. Anthraquinones give red color, anthrones color yellow and coumarines color blue at 365 nm. About 10 g solid iodine is spread on the bottom of a chromatography tank. Compounds containing conjugated double bonds give yellow-brown (vis.) zones on exposure to the atmosphere of iodine vapor. The TLC plates were sprayed with a solution of 37% formaldehyde in conc. sulfuric acid (1:10) immediately after taking from developing chamber. The presence of various spots on the plates detected alkaloids, aromatic hydrocarbons, e.g., antihypertensive drugs.

3.20. Disc diffusion method

The disc diffusion method was used to study the antibacterial activity of the crude extracts against test pathogens (Bauer *et al.*, 1966). The organic extract with a concentration of 30 µg was prepared and incubated onto the sterile discs. The pathogenic bacteria were swabbed onto MHA pates. The discs were then placed on the center of the MHA plates and incubated at 37 °C for 24–72 h. Antibacterial activity was measured as the diameter of the zone of inhibition using antibiotic zone scale (HiMedia). Chloramphenicol (HiMedia) was used as positive control and ethyl acetate as negative control.

3.21. Minimum inhibitory concentration and minimum bactericidal concentration

The MIC was determined in a 96 well micro-plate. A total of 8 test pathogens and the organic extracts were added in different dilutions. An 18h old pathogenic bacterial cultures (100 µL) and organic extract (100 µL) was added to each well in different concentrations. Nutrient broth was set as blank, chloramphenicol as the positive control and ethyl acetate as the negative control. The microplate was incubated for 18 h at 35 °C. A tetrazolium salt, MTT (5 %) was used to determine the growth of the bacteria as MTT stains live cells in purple indicating their growth. The concentration in which the growth was not exhibited (no color change), was found to be the MIC of the organic extract. The minimum bactericidal concentration (MBC) was determined by swabbing 10 µL of the dilutions from the incubated plates to spot the colony formation, and the lowest concentration at which the colony formation was not observed, were recorded.

3.22. Bioassay of the organic extract from *Bacillus velezensis* MBTDLP1 associated with marine macroalgae

3.22.1.In *vitro* antioxidant assays

3.22.1.1. 2, 2'-Diphenyl-1-picryl hydrazil (DPPH•) radical scavenging activity

The organic extract was prepared in different concentrations (0.125 to 2 mg/mL). The various concentrations of extracts (1 mL) were added to equal volumes of 0.1 mM DPPH in methanol (1 mL). The solutions were kept at RT in the dark for 20 min. The DPPH solution in methanol served as the control. The absorbance of various concentrations of extracts and control solutions were recorded against methanol as blank after 20 min at 517 nm using UV-VIS spectrophotometer. The triplicate experiments were carried out. The percentage inhibition values were plotted against concentration of the extract to deduce the IC_{50} value radical scavenging activity of the organic extract. The percentage inhibition (%) was calculated as follows: $((A_C - A_S)/A_C) * 100$, where A_C and A_S represented the absorbance of control and sample, respectively.

3.22.1.2. 2 -Azino-bis-3-ethylbenzothiozoline-6-sulfonic acid diammonium salt ($ABTS^+$) radical scavenging activity

In brief, $ABTS^+$ (35 mg in 10 mL) and potassium persulfate (6 mg in 10 mL) were mixed by sonication and kept in dark at room temperature for 16 h. The blue coloured $ABTS^+$ stock solution was then diluted with MeOH to get ~0.70 absorbance at 734 nm. The organic extracts were prepared in various concentrations (0.125- 2 mg/mL). The diluted $ABTS^+$ (1mL) was mixed with 1 mL of extracts and their corresponding absorbance was recorded at 734 nm against blank solution of methanol using a UV-VIS spectrophotometer. The triplicate analyses were performed. The percentage inhibition (%) was calculated as follows: $((A_C - A_S)/A_C) * 100$, where A_C and A_S represented the absorbance of control and sample, respectively. The percentage inhibition was

plotted against the concentration of the extracts to determine IC_{50} value for scavenging activity of the extract.

3.22.1.3. Ferrous ion chelating ability

Ferrous ion chelation assay was carried out for organic extracts of *B. velezensis* MBDLP1 for determining their metal ion chelating potential (Gulcin, 2007). Briefly, the organic extracts (0.125-2 mg/mL) were added to a solution of $FeSO_4$ (1 mL, 0.125 mM) and ferrozine (1.0 mL, 0.3125 mM). The mixture was vortexed and incubated for 10 min at RT. The absorbance was measured at 562 nm. % Fe^{2+} ion chelating ability = $(A_0 – A_1) \times 100 / A_0$, where A_0 is the absorbance of control and A_1 is the absorbance of sample. The IC_{50} values were deduced by plotting percentage chelation against concentrations of the organic extract.

3.22.2. in vitro anti-inflammatory assay

In vitro 5-LOX assay was carried out to determine the anti-inflammatory activity of the organic extract of *B. velezensis* MBTDLP1. The extracts were prepared in mixture of DMSO and Tween 20 in the ratio 29:1 in various concentrations from 0.125-2 mg/mL. In a 96 well plate, 50 µL of sample along with 30 µL linoleic acid, 30 µL of 5-LOX enzyme (prepared in potassium buffer) and 18 µL of potassium buffer was added. The control was served by the mixture of DMSO: Tween 20 followed by linoleic acid, potassium buffer, and enzyme and DMSO: Tween 20 mixture was taken as blank. The absorbance was taken at 234 nm. The IC_{50} value (mg/mL) was calculated from the plotted curve of the results of percentage inhibition against concentration.

3.22.3. *in-vitro* antidiabetic assays

3.22.3.1. α-Amylase inhibition assay

For the assessment of inhibition of the carbolytic α-amylase enzyme, organic extracts prepared in phosphate buffer (500 µL, 0.20 mM, pH 6.9). The enzyme α-amylase (0.5 mg/mL) was prepared in buffer and added to fractions of extracts. The solution was incubated at 25°C for 10 min. Following, starch solution (500 µL) was added and incubated at 25°C for 10 min. The reaction was stopped with 3, 5 dinitrosalicylic acid reagent (DNS) (1.0 mL) under heating for 5 min and cooled to room temperature. The absorbance was measured at 540 nm after diluting the mixture with distilled water. The control carries 100 % α-amylase activity and phosphate buffer served as

blank reagent. The plot of inhibition of α-amylase activity was recorded to calculate the IC_{50} value (mg/mL).

3.22.3.2. α-glucosidase inhibition assay

For *α-glucosidase* assay, the organic extracts were prepared in Tris-HCl buffer (500 µl, 0.2M, pH 8) in different concentrations, and were added to the enzyme solution (1 U/mL prepared in 0. 2M Tris-HCl, pH-8). The reaction mixture was then incubated at 37°C for 5 min. Starch solution (500 µL, 2 %, w/v) was added and incubated for 10 min at 37°C. The reaction was stopped with 3, 5 dinitrosalicylic acid reagent (1 mL) under heating for 2 min in a boiling water bath before being cooled at room temperature. The reaction mixture was then diluted with distilled water (9 mL). The absorbance of the reaction mixture was measured at 540 nm. The IC_{50} value was determined from the plotted graph of percentage inhibition against concentrations used.

3.22.4. Determination of anti-cancerous properties

Ex-vivo studies were done to determine the anti-cancerous properties of the organic extract. The cancer cell lines used in the study were MCF-7 (Human breast cancer cell line) and 3T3L cell line (Fibroblast cell line) as normal cell reference. Doxorubicin was used as standard drug reference in comparison to organic extract and untreated cell line was used as control. The media without any cells were treated as blank.

3.22.4.1. Cytotoxicity assays: (A) MTT assay

For determining the cytotoxicity effect of the organic extract, ex-vivo studies were carried out in cancer cell lines. A cell suspension of 200µl was seeded in a 96-well plate at required cell density (20,000 cells per well), without the test agent. The cells were allowed to grow for about 24 hours. Further, extracts were added to appropriate concentrations (12.5µg, 25 µg, 50 µg, 100 µg, 200 µg) into the plates and incubated for 48 hours at 37°C in a 5% CO2 atmosphere. After the incubation period spent media was removed and MTT reagent was added to a final concentration of 0.5mg/mL of total volume. The plate was incubated for 3hours in dark. MTT reagent was removed and 100 µl of solubilisation solution (DMSO) was added. It was gently stirred in a gyratory shaker to enhance dissolution. The absorbance was taken on the ELISA reader at 570 nm and 630 nm used as reference wavelength. The IC_{50} value was determined by using linear regression equation i.e. Y =Mx+C. Here, Y = 50, M and C values were derived

from the viability graph. The percentage of cell viability was calculated using the formula

Table 3.2. Concentrations of the test compounds used for the study.

Sl.No	Test Compound	Cell Line	Concentration treated to cells
1	Untreated	MCF7 & 3T3-L1	No treatment
2	Doxorubicin	MCF7 & 3T3-L1	5ug/mL
3	Blank	-	Only Media without cells
4	AF-4	MCF7 & 3T3-L1	12.5, 25, 50, 100, 200µg/m

3.22.4.2. Cytotoxicity assays: (B) NRU assay

In a 96 well plate, the cells were seeded at required cell density without the test reagent. The cells were allowed to grow for about 24 hours. Further, extracts were added to appropriate concentrations (12.5µg, 25 µg, 50 µg, 100 µg, and 200 µg) into the plates and incubated at 37°C for 48 hours in a 5% CO2 atmosphere. After the incubation period, spent media was removed and 100ul of Neutral Red staining solution was added. The plate was and incubated for 2 hours in dark (Note: Incubation time varies for different cell lines. Within one experiment, incubation time should be kept constant while making comparisons.). Neutral Read reagent was removed and 200 µL of washing solution (DPBS) was added carefully to remove PBS without disturbing monolayer of cells. Subsequently, 150 µL of DMSO was added to each well. Gentle stirring was done in a gyratory shaker to enhance dissolution of the Neutral Red reagent. The absorbance was read on an ELISA reader at 540nm and 630nm used as reference wavelength.

Table 3.3. Concentrations of the test compound used to treat the cell lines

Sl.No	Test Compound	Cell Line	Concentration treated to cells
1	Untreated	MCF7 & 3T3-L1	No treatment
3	Doxorubicin	MCF7 & 3T3-L1	5ug/mL
3	Blank	-	Only Media without cells
4	AF-4	MCF7 & 3T3-L1	12.5, 25, 50, 100, 200µg/mL

3.22.4.3. Apoptosis assay

The cells were cultured in a 6-well plate at a density of 0.5 x 10^6 cells/2 mL and incubated overnight at 37°C for 24 hours in a CO_2 incubator. The spent medium was removed and the cells were treated with organic extract of different concentrations along with controls, in 2 mL of culture medium and incubated for 48 hours. The medium was removed and washed with PBS. After discarding PBS, a 200 µL of trypsin-EDTA solution was added. The plate was incubated for 3-4 minutes at 37°C. The cells were directly harvested into 12 x 75 mm polystyrene tubes after the addition of culture medium. The tubes were centrifuged at 300 x g at 25°C for five minutes. The supernatant was discarded carefully and the cells were washed twice with PBS. A 5 µL of FITC Annexin V was added and the plate was gently vortexed and incubated at RT (25°C) in the dark for 15 min. A 5 µL of Propidium Iodide (PI) and 400 µl of 1X Binding Buffer were added to each tube and vortexed gently. Analysis by flow cytometry was done immediately after addition of PI.

Table 3.4. Concentrations of the compound to treat the cell lines

Sl.No	Test Compounds	Cell Line	Concentration treated to cells
1	Untreated	MCF7 & 3T3L1	No treatment
2	Standard (doxorubicin)	MCF7 & 3T3L1	10µg/mL
3	Test (AF4)	MCF7	32.22µg/mL
4	Test (AF4)	3T3L1	140.13µg/mL

3.22.5. Spectroscopic fingerprint analyses

Spectroscopic fingerprint analyses of the organic extract of *B. velezensis* MBTDLP1 was comprehensively studied by nuclear magnetic resonance (^{1}H NMR) spectral analysis on a Bruker AVANCE III 600 MHz spectrometer (Bruker, Germany). Chemical shift (δ_H) values were recorded as ppm (δ, parts per million), and the protons at the definite regions of the ^{1}H NMR spectrum were integrated (proton integral, Σ*H*) to determine the aggregate number of protons in characteristic regions of the spectrum. The ^{1}H NMR spectrum were allocated into five discrete regions, such as alkane hydrocarbon (primary through tertiary, δ_H 0.1–2.0), allylic

(CH_2=CH– Me)/acetyl {–C(=O) Me}/derivatized hydride of alkanoates {–CH_2C(=O)O–, δ_H 2.1–2.5}, methoxyl (–OMe)/functionalized alkanol (–CH_2OH, δ_H 2.6–3.5), anomeric (due to polysaccharides, δ_H 3.6–4.5), olefinic (–CH=CH–)/protons of the hydride group of alkanoates

{CH_2C(=O) OMe, δ_H 4.6–6.0}, and aryl protons (C_6H_5–H, δ_H 6.6–8.6. The ^{1}H NMR trace at δ_H 9–10 was characterized by the aldehydic protons.

3.22.6. Statistical analysis

One-way analysis of variance (ANOVA) was carried out for the statistical analyses to assess the differences between the means of bioactivities. The differences were represented as $p < 0.05$, and the values were presented as the means of triplicate ± standard deviation.

RESULTS

4. RESULTS

4.1. Sample Collection

Marine algae belonging to Phaeophyceae and Rhodophyceae were collected from the Southwest Coast of Peninsular India. The main collection sites included Mandapam, Vizhinjam.

4.2. Isolation of marine algae associated heterotrophic bacteria

Marine algae belonging to Phaeophyceae and Rhodophyceae along the Southeast coast of India, were collected and the bacteria associated with them were isolated and screened for bioactive potentials. Bacteria from 8 different macroalgae viz., *Sargassum wighti, Gracilaria edulis, Turbinaria ornata, Padina pavonica, Sargassum tenerrium, Sargassum marginatum, Laurencia papillosa*. The serially diluted (from 10^{-1} to 10^{-7}) samples were plated in NA supplemented with 1% NaCl (Figure 4.1). The plates were observed for growth up to 1 week. Well grown isolates and isolates that showed some inhibition pattern were selected and subcultured. A total of 40 bacterial isolates were isolated (Figure 4.2).

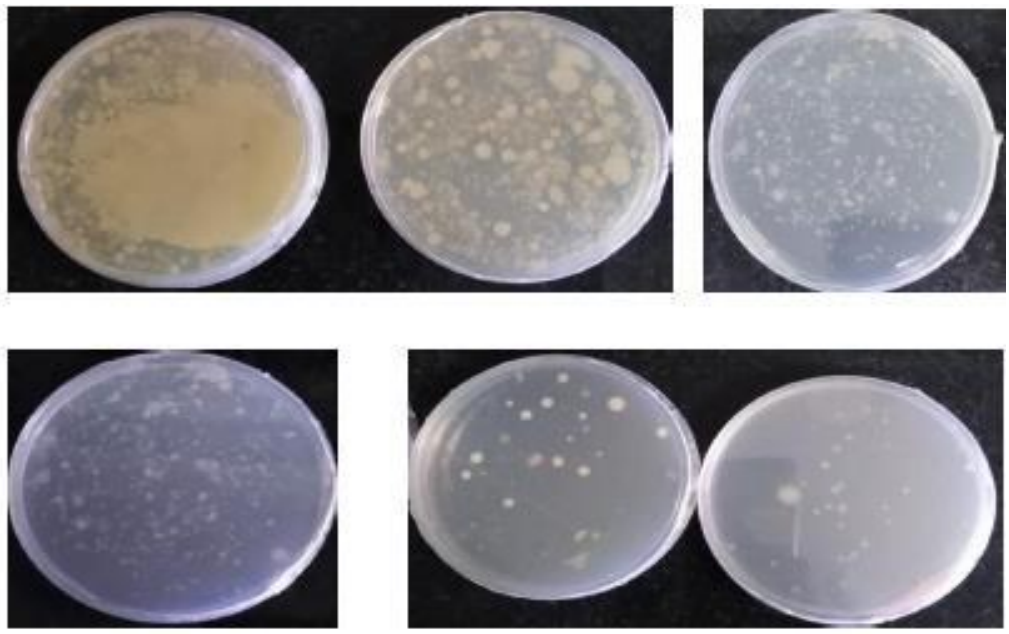


Figure 4.1. Serial dilutions from 10^{-1} to 10^{-6}

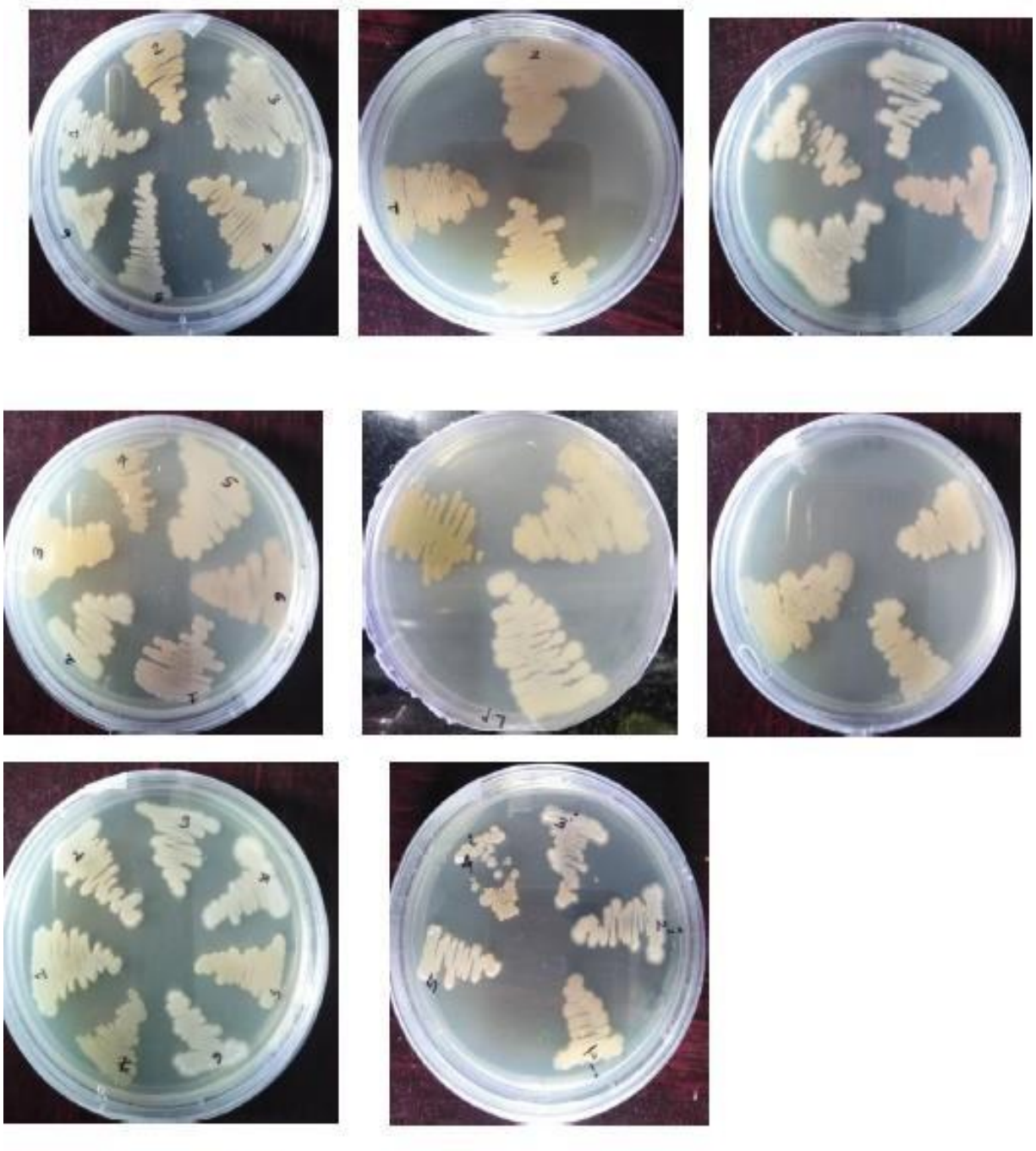


Figure 4.2. Isolates from marine algae

The distribution of the microbial diversity among collected marine algae was computed in which the most number of isolates were contributed by *S. wightii* (20%), followed by *S. tennerimum, T. ornate, P. pavonica, S. marginatum* each with 12.5% of the isolates. The marine algae *L.*

papillosa and G. salocornia gave 10% of isolates while *G. edulis* contributed the remaining isolates (Figure 4.3).

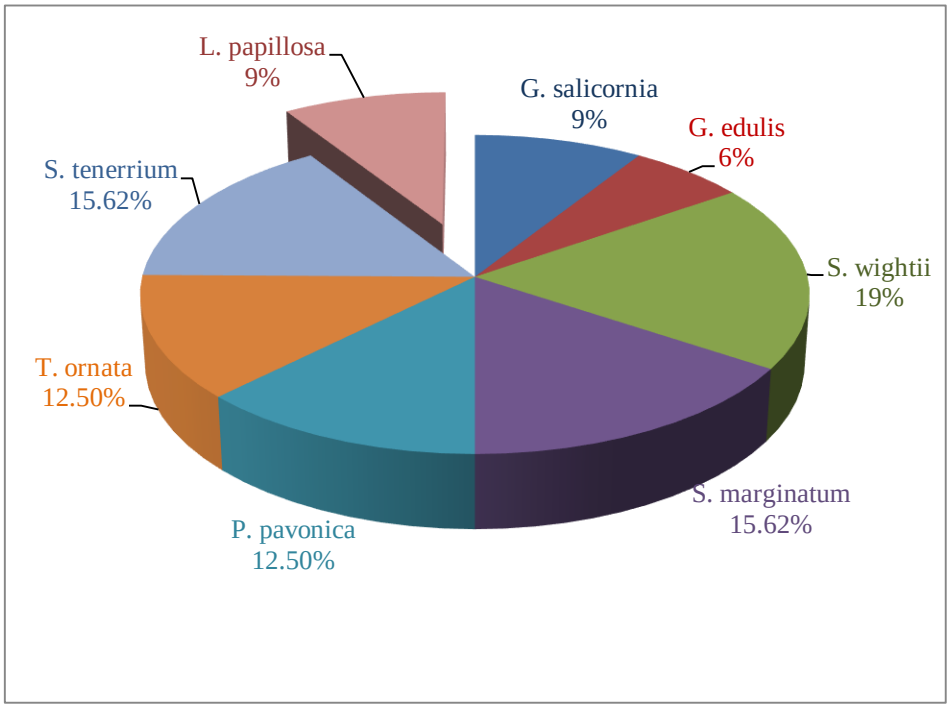


Fig 4.3. Pie chart distribution of marine algae associated heterotrophs from the screened marine algal samples.

4.3. Preliminary antibacterial activity screening

The 40 isolates were screened by spot-over-lawn assay against different test pathogens, *Vibrio parahaemolyticus* (MTCC 451), *Aeromonas salmonicida* (ATCC 27013), *Photobacterium phosphoreum* (MTCC11040), *Escherichia coli* (MTCC 443), *Streptococcus pyogenes* (MTCC 1924), *Edwardsiella tarda* (MTCC 2400), *Aeromonas caviae* (ATCC 15468) and methicillin resistant *Staphylococcus aureus* (MRSA). The zone of inhibition of diameter was measured in terms of millimeters. A total of 8 isolates showed antagonist activity against some of the pathogens used in the study. Among 8 isolates, 4 showed against *Vibrio parahaemolyticus*, 6 of them showed activity against *Yersinia enterolytica* and 7 of them showed activity against *Escherichia coli*. Only 2 of the isolates showed activity to human pathogens, *Streptococcus pyogenes* and methicillin resistant *Staphylococcus aureus* (Table 4.1) (Figure 4.4). Two of the isolates that were

included in all the statistics were selected as most active isolates and characterized further by biochemical and molecular characterization.

Table 4.1. Antimicrobial spectrum of the selected isolates by spot-over-lawn-assay

Strains	*Vibrio parahemolyticus* (MTCC 451)	*Aeromonas salmonicida* (ATCC 27013)	*Yersinia enterocolitica* (MTCC 859)	*Escherichia coli* (MTCC 443)	*Streptococcus pyogenes* (MTCC 1924)	*Edwardsiella tarda* (MTCC 2400)	*Aeromonas caviae* (ATCC 15468)	*S. aureus* (MRSA) MTCC 33952
SM1	7.3±0.47	ND	12.6±0.43	7±0.24	ND	8±0.24	ND	ND
SW1	ND	ND	17.6±0.43	ND	ND	8±0.16	ND	ND
TO2	ND	ND	14.5±0.4	7±0.16	ND	ND	ND	ND
LP1	35±0.81	7±0.29	20±0.81	12±0.23	20.3±0.62(B)	12±0.4	10±0.81	36±0.47
LP2	17±0.81	7±0.84	11±0.61	7±0.32	18±0.81(BS)	11±0.62	7±0.4	32±0.29
GE1	ND	ND	ND	7±0.47	ND	ND	ND	ND
ST1	7.6±0.47	ND	ND	7±0.32	ND	ND	ND	ND
ST2	ND	ND	12.5±0.4	7.5±0.4	ND	8±0.62	ND	ND

The samples were analyzed in triplicates (n = 3) and expressed as mean ± standard deviation.

ND- Not detected any significant inhibition;

BS- Bacteriostatic zone

4.4. Screening by antioxidant activity

The preliminary screening of the antioxidant activity of the isolate was assessed by the filter paper incubation method against DPPH. The zone of clearance was measured in the filter paper after spraying the DPPH into it. The selected two strains showed zone of clearance against DPPH radical with zone diameter ranging from 12-15 mm representing radical scavenging activities.

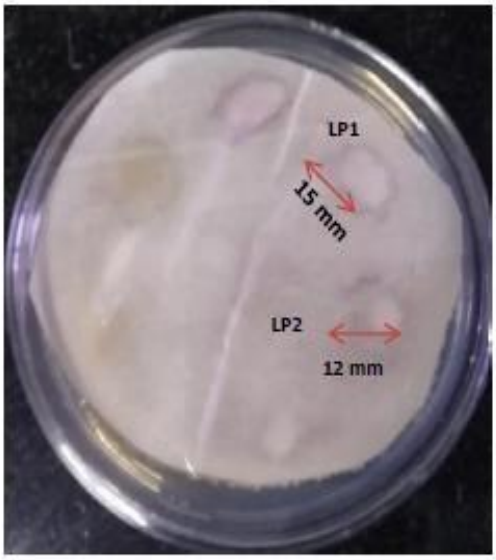


Figure 4.4. Filter paper representing the zone of clearance against DPPH radical by the isolates LP1 and LP2.

4.5. Biochemical characterization of the isolates

The two isolates which exhibited significant antagonistic properties in the preliminary screening were identified by biochemical tests in line with Bergey's Manual of Determinative Bacteriology protocol (Chakraborty *et al.*, 2014). Further biochemical tests were achieved by HiMedia HiBacillus identification kit (KB013). The isolates were Gram-positive with short-chained spores (Figure 4.5). The catalase positive, aerobic, isolates showed motility and growth in different NaCl concentrations and optimum growth in 34 °C. The tests were positive for starch hydrolysis, citrate utilization, oxidase activity, voges-proskauer, nitrate reduction, arginine utilization and carbohydrate utilization. Negative results corresponded for malonate utilization, beta-galactosidase, indole production, and lysine decarboxylation (Table 4.2). The Gram-positive isolates that showed positive catalase reactive were aerobic bacteria with spore forming and carbohydrate utilization. These observations categorized them under the genus *Bacillus* spp. In addition to this, the isolates showed motility, gelatin liquefaction, and starch hydrolysis (Figure 4.6). Their species level was identified by molecular characterization using 16s rRNA sequence analysis.

Figure 4.5. Biochemical tests performed using HiMedaa Hi*Bacillus* Identification kit (KB013). (A).Control, (B) LP1 and (C) LP2.

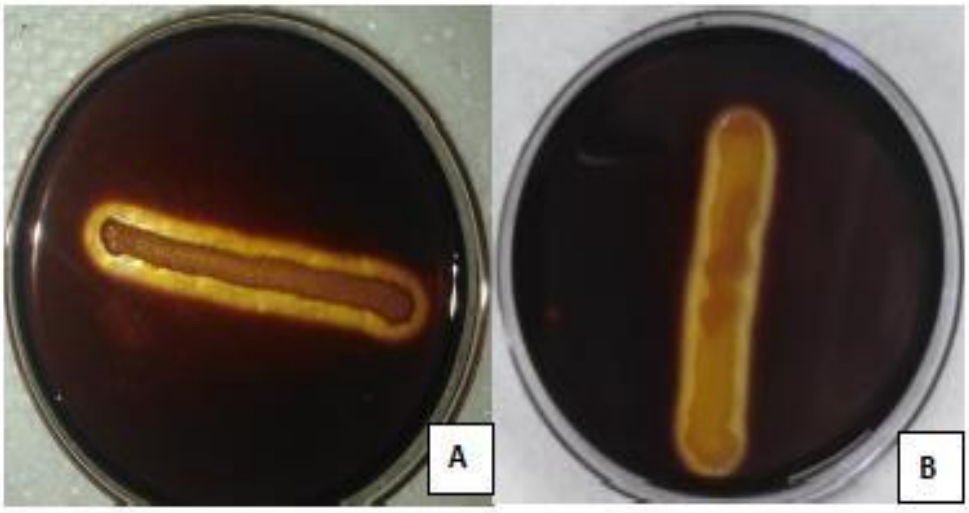


Figure 4.6. Starch hydrolysis of the selected isolates (A) LP1 and (B) LP2

Table 4.2. Biochemical characterization of the selected isolates

Sl. No	Biochemical Test	LP1	LP2
1	Gram Staining	+	+
2	Optimum Temperature	37º C	37º C
3	Growth in 7% NaCl	+	+
4	Malonate*	-	-
5	Voges- Proskauers*	+	+
6	Citrate utilization*	+	+
7	Nitrate reduction*	+	+
8	Beta galactosidase*	-	+
9	Catalase*	+	+
10	Arginine*	+	-
11	Sucrose*	+	+
12	Mannitol*	+	+
13	Glucose*	+	+
14	Arabinose*	+	+
15	Trehalose*	+	+
16	Gelation liquefaction	+	+
17	Oxidase	+	+
18	Indole production	-	-
19	Motility	+	+
20	Lysine decarboxylation	+	-

‘+’ denotes positive reaction of the isolate to the specific biochemical test, ‘- ‘denotes negative reaction. * denotes biochemical tests from HiBacillus identification kit KB013.

Table 4.3. Morphological characteristics of the test bacteria

Tests	LP1	LP2
Colony Morphology		
Configuration	Wrinkled	Wrinkled
Margin	Rhizoidal	Rhizoidal
Elevation	Flat	Flat
Opacity	Opaque	Opaque
Surface	Rough	Rough
Pigment	Off-white	Off-white
Gram's reaction	+	+
Cell shape	Rods	Rods
Arrangement	Chains	Chains
Spores	+	+
Shape	Ellipsoidal	Ellipsoidal
Motility	+	+

(+) denotes positive reaction for the test and (–) denotes negative.

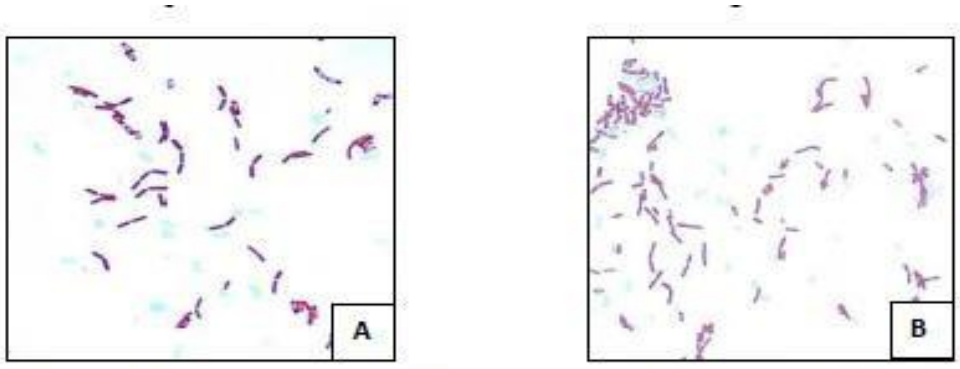


Figure 4.7. Indicative pictures of Gram staining of the isolates (A) LP1 and (B) LP2. Gram positive strains of *Bacillus* genus

4.6. Molecular characterization of the selected isolates

The genomic DNA was isolated by the GenElute Bacterial Genomic DNA isolation kit. The DNA isolates with and without RNase treatment was run in agarose gel electrophoresis to check the quality and purity of the DNA retrieved. The DNA treated with RNase showed a distinct band against the ladder used (Figure 4.7, lane 1 and 3). The separation of bands of the ladder ensured the accurate mobility in the electrophoresis unit. The DNA without RNase treatment showed smeared band indicating the RNA contamination. The ratio of the absorbance at $A_{260/280}$ gave the values 1.7 and 1.75 that give the quality check of a good DNA. The concentration of the DNA samples was determined as 65.6 ng/µL for LP1 and 55.2 ng/µL for LP2. The samples were stored at 4 ^{0}C until further proceedings.

The 16S rRNA gene amplification gave amplicons of 1500 bp. The amplicons were sequenced by forward primer and retrieved sequences were aligned with the similar hits in GenBank and accordingly, a phylogenetic tree was created. Evolutionary analyses were conducted in MEGA X. The BLAST similarity searches showed that the isolates showed 99 % similarity with the closest relatives. The isolates from *Firmicutes* phyla converged onto *Bacillus* genus by biochemical tests and into species level identification by 16S rRNA gene sequencing that resulted in *Bacillus velezensis* and *Bacillus altitudinis* which was later named as *B. velezensis* MBTDLP1(LP1) and *B. altitudinis* MBTDLP2 (LP2).

Screening of the functional genes in the isolates was carried out with the specific primers tagging the biosynthetic genes in the DNA. Primers specific for *pks* and *nrps* genes were amplified. An amplicon of size 700 bp marks the positive amplification of *pks-I* gene in the genome and an amplicon of 1000 bp marks the presence of *nrps* genes in the genome assembly. The amplicons were further sequenced against forward primer. The sequences were then checked for the similarity and type of secondary metabolite present. The bacterium, *B. velezensis* showed positive pks amplification *B. altitudinis* failed to amplify. No *nrps* gene was amplified for both isolates. Thus, the Gram-positive, *pks*-positive isolate, *B. velezensis* was selected for further bioprospecting studies.

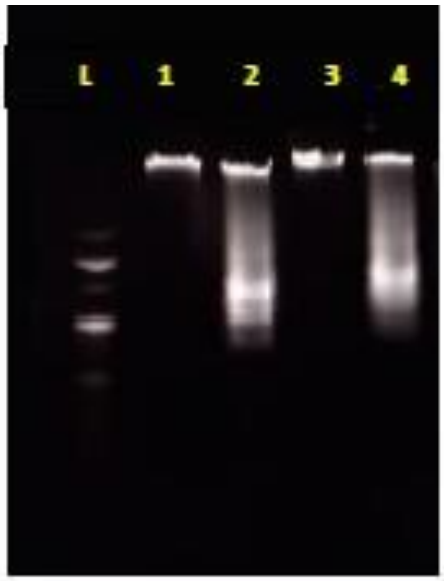


Figure 4.8. Gel profile of the DNA isolated from the selected bacterial strains LP1 and LP2. The lane L denotes the ladder (1Kb, HiMedia) and isolated DNA of the bacterial samples LP1 and LP2 denotes from lane 1-4 (lane 1, 3 denotes DNA without RNase treatment).

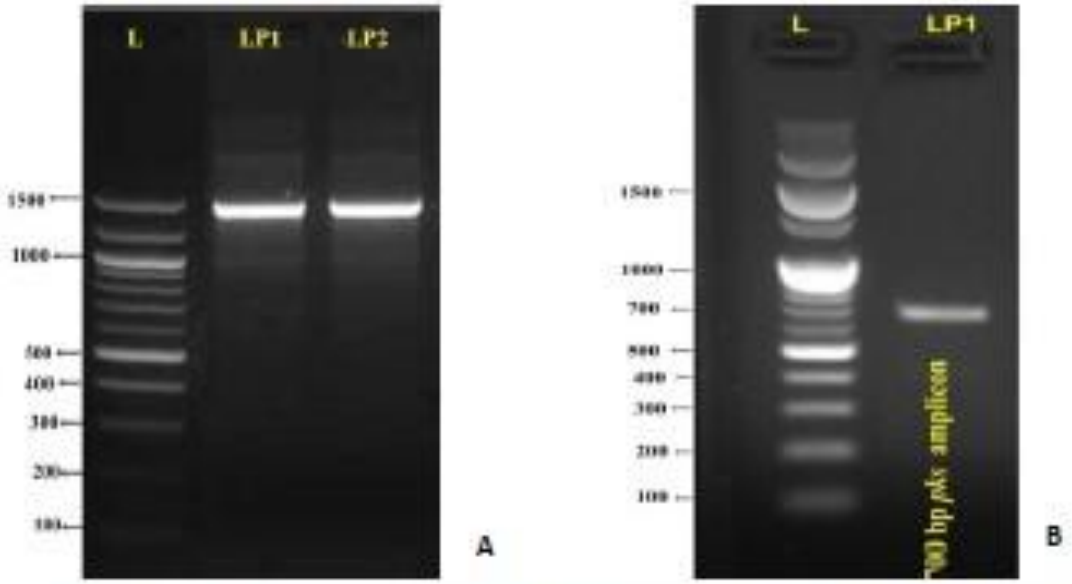


Figure 4.9. Gel profile of the isolates (A) 16S rRNA gene amplification with amplicons of size 1500 bp. The lane L denotes the ladder (1 Kb, HiMedia) and Lane LP1 and LP2 denote the selected isolates. (B). Gel profile of type-I pks amplification of the isolate LP1 showing positive amplicons of 700 bp. The Lane L denotes ladder (1Kb, HiMedia) and LP1 denotes the isolate.

The 16S rRNA gene sequences were submitted in NCBI GenBank with accession number MT122835 (LP1) and MT122905 (LP2). The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-4100.03) is shown. 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 Tamura-Nei model, 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. This analysis involved 10 nucleotide sequences. There were a total of 1513 positions in the final dataset. (Kumar *et al*. 2018).

Type-1 polyketide synthase gene was amplified in the candidate bacterium, *B. velezensis* MBTPLP1 and the sequence showing significant homology in BLAST search was submitted in NCBI GenBank with accession number MT394492. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones *et al*. 1992). The tree with the highest log likelihood (-7532.22) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 9 amino acid sequences. There were a total of 493 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*. 2018). There was no specific amplification detected for the *nrps* primers used.

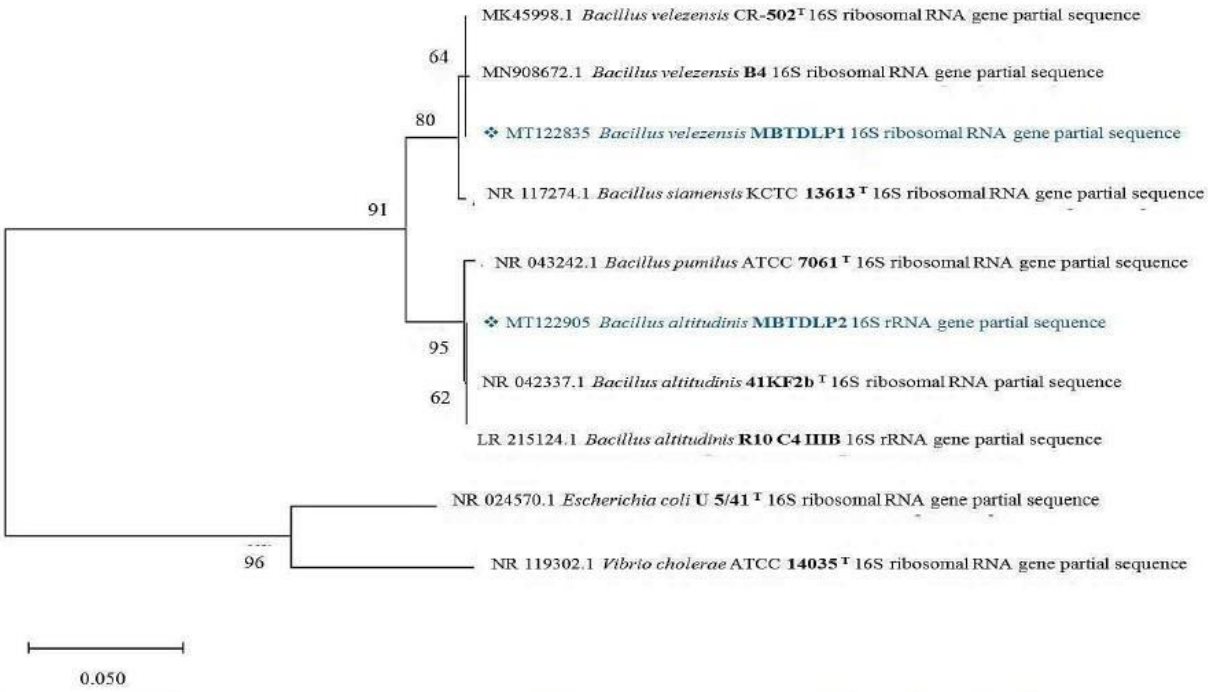


Figure 4.10. Phylogenetic analysis of 16S rRNA gene sequence of the selected isolates LP1 and LP2 along with the relative species

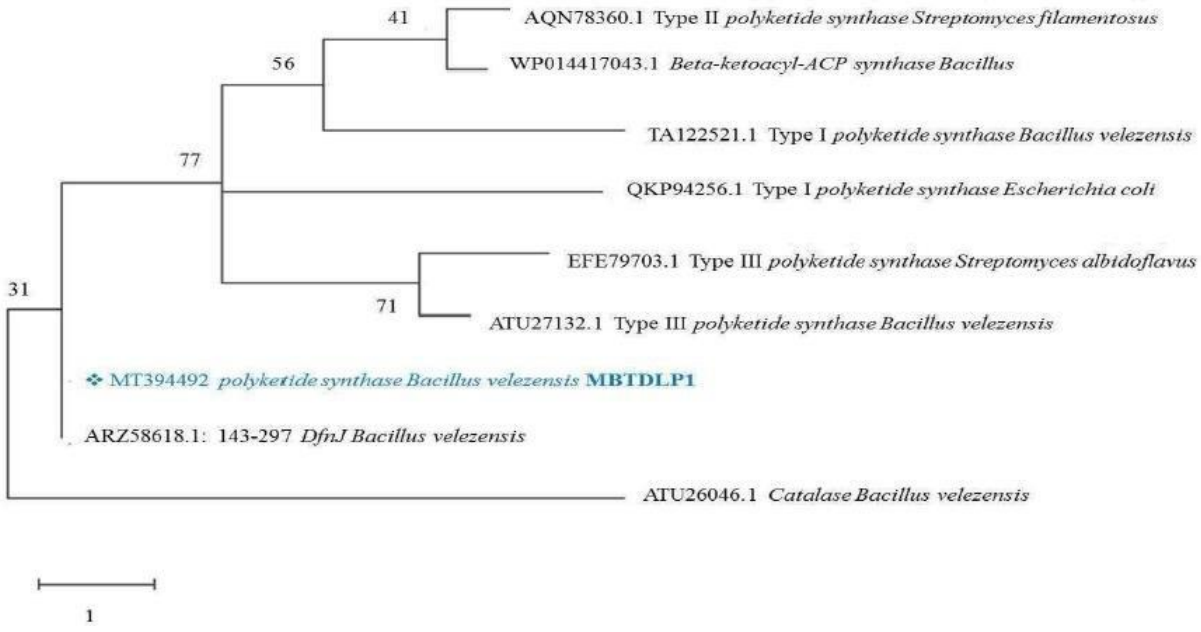


Figure 4.11. Phylogenetic analysis of *pks-I* gene sequence of the isolate *B. velezensis* MBTDLP1

4.7. Antibiotic Susceptibility of the isolate *B. velezensis* MBTDLP1

The selected isolate showed susceptibility to the commercially available antibiotics expressed in Octadiscs (HiMedia) and other antibiotics. The antibiotics collectively showed inhibition against the selected isolate reporting that the bacterium is susceptible and does not pose any threat of resistance to available antibiotics.

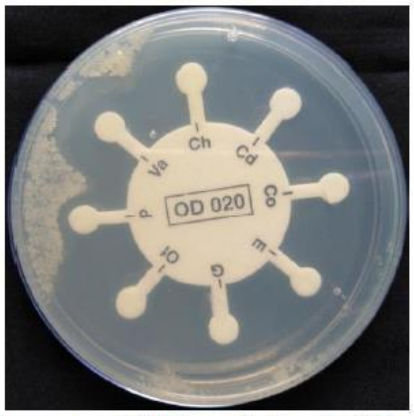


Figure 4.12. Antibiotic susceptibility of the isolate against commercially available antibiotics expressed as Octadiscs (HiMedia)

4.8. Siderophore production by the marine *Bacillus*

Siderophore production was marked using CAS agar detection for the production of yellow clear zone. The plates were observed for the growth of culture up to one week. The growth was marked by the yellow colored zone from the border of the spotted culture. A yellow zone of diameter (25 mm) was obtained corresponding to the CAS assay denoting the positive results for siderophore production.

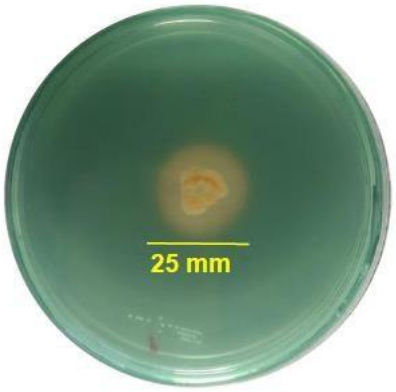


Figure 4.13. Siderophore production by *B.velezensis* MBTDLP1 on CAS agar plate with zone of diameter 25 mm

4.9. Bacterial growth curve of *B. velezensis* MBTDLP1

Bacterial communities produce secondary metabolites mainly in their stationary phase of growth. The growth kinetics plotted for the selected bacteria, *B. velezensis* MBTDLP1 shows the stationary phase at 68-72 hours of the growth. To add on the result, the maximum zone of diameter of inhibition on spot-over lawn assay corresponded to the third day of incubation, i.e. from 68 to 74 hours. Extraction from the bacteria was carried out based on the stationary phase from the growth curve.

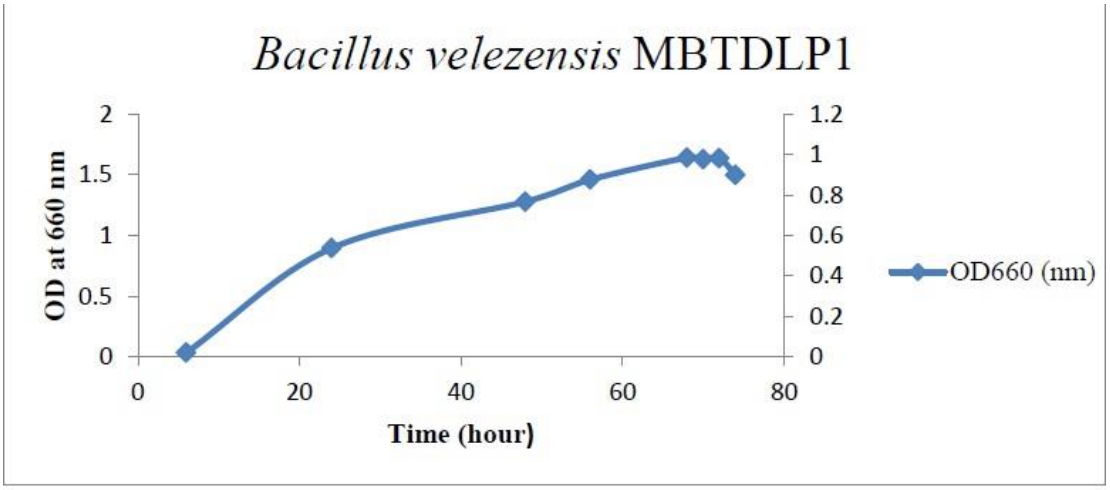


Figure 4.14. Bacterial growth curve of *B. velezensis* MBTDLP1 at 660 nm.

4.10. Extraction of bioactive compounds from *B. velezensis*

The most active and *pks* positive *B. velezensis* was selected for further bioprospecting studies. The bacterium was grown on nutrient broth supplemented with 1% NaCl and subsequently swabbed onto nutrient agar with 1% NaCl. The cells and agar were separated and extracted individually for extracellular and intracellular metabolites. The bioactive metabolites were extracted using the solvent ethyl acetate and was exhaustively refluxed for 6 days. Ultrasonication derived intracellular metabolites and extracellular organic extract was documented using thin layer chromatography to determine the presence of compounds.

4.11. Thin Layer Chromatographic documentation and visualization of the organic extract

The organic extract showed good separation on the solvent system 100% ethyl acetate. The TLC plates were documented. Presence of UV detectable spots was analyzed in 254 nm (5 spots) and 365 nm (6 spots) indicating the presence of conjugated and aromatic rings. Visualization with iodine gave 5 spots indicating the presence of unsaturated or aromatic compounds due to its high affinity. A total of 3 spots were documented for alcoholic KOH detecting the presence of anthrones that give yellow colour in 365 nm. One spot was obtained for sulphuric acid-formaldehyde indicating the presence of alkaloids or aromatic hydrocarbon.

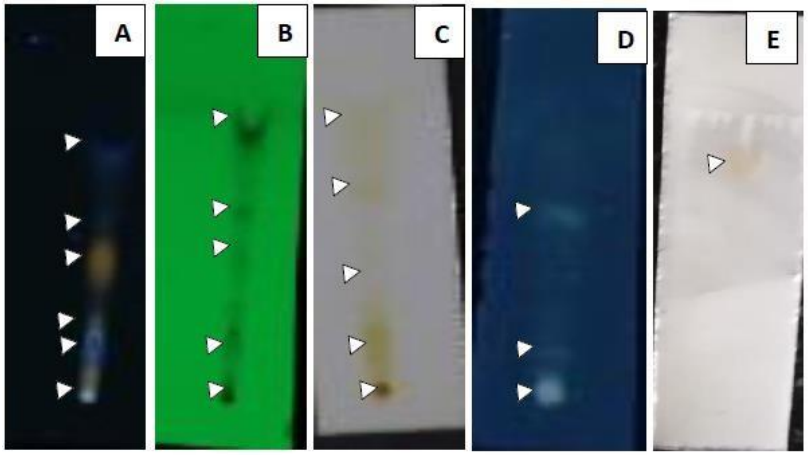


Figure 4.15. TLC visualization of the extracellular extract of *B. velezensis* MBTDLP1 (LP1) with (A) UV visualization at 365 nm (B) UV visualization at 254 nm (C) Staining with iodine (D) with alcoholic KOH and (E) with formaldehyde in sulphuric acid.

4.12. Anti-bacterial activity of the organic extract of *B. velezensis* MBTDLP1 and their minimum inhibitory concentration

The extracellular metabolites of the selected bacteria *B. velezensis* MBTDLP1 was extracted using the solvent ethyl acetate. A yield of 5 g was concentrated and stored according to the established protocol. The antagonistic property of the organic extracts of *B. velezensis* MBTDLP1 was assessed by disc diffusion assay and minimum inhibition concentration. The zone of inhibition from disc diffusion assay and the minimum inhibition concentration were

summarized in Table 4.4. The organic extract displayed well promising results in the disc diffusion assay against the test pathogens, *V. parahaemolyticus, A. salmonicida, Y. enterolytica, E. coli, S. pyogenes, E. tarda, A. caviae* and methicillin resistant *S. aureus* (MRSA). The antagonist activity showing 25 ± 0.40 mm of zone against MRSA ATCC 33952 followed by 20± 0.29 mm against *S. pyogenes* MTCC 1924 (Figure 4.16) was observed. It was observed that the MIC of the organic extract of *B. velezensis* MBTDLP1 was 7.5-30 µg/mL respectively. The results showed consistency up to one week further after observation. The organic extracts of *B. velezensis* MBTDLP1 exhibited strong antibacterial activity against a broad spectrum of pathogenic bacteria, *E. coli, S. pyogenes, E. tarda, V. parahemolyticus,* MRSA. The microdilution method also displayed a significant MIC of 7.5–30 µg/mL against the test pathogenic bacteria (Table 4.4).

The standard chloramphenicol showed antagonist activity with a zone of inhibition of diameter 15 mm against MRSA, 11 mm against *V. parahaemolyticus,* 10 mm against *A. salmonicida, E. coli* and 9 mm against *A. caviae* and 8 mm against *E. tarda* and no zone against *S. pyogenes* and
V. parahaemolyticus ATCC 17802. The MIC of the standard was shown as 6.25 µg/mL.

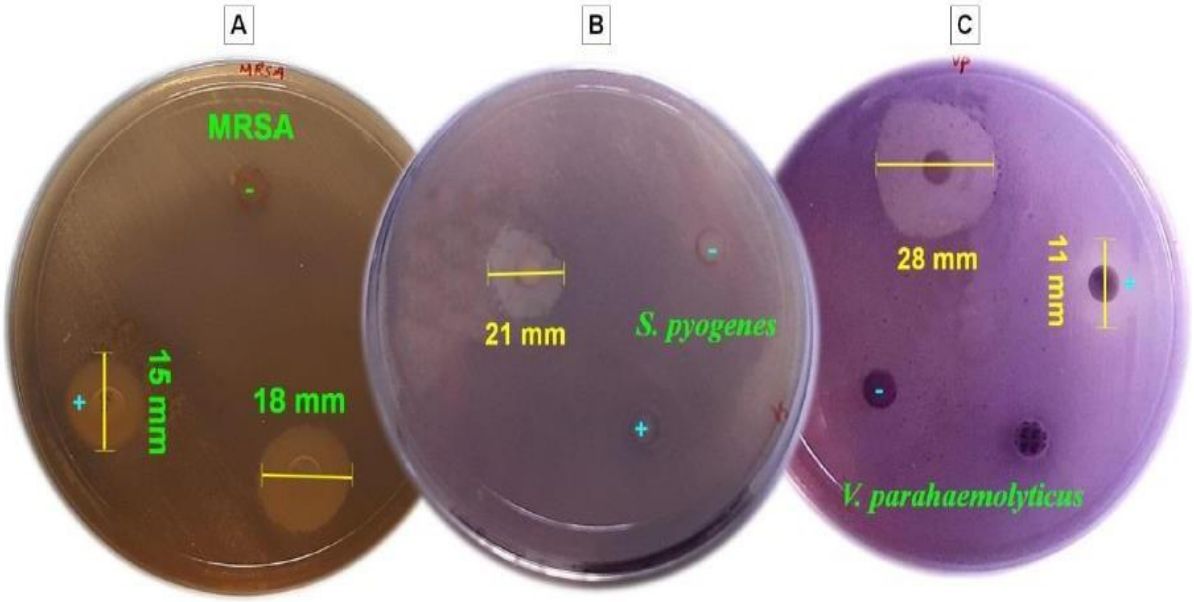


Figure 4.16. Anti-bacterial activity of the organic extract of B.velezensis MTDLP1 against

(A) *V.parahemolyticus,* (B) *S. pyogenes* and (C) MRSA.

Table 4.4. Antibacterial activities of intertidal macroalga-associated *B. velezensis* MBTDLP1 against pathogenic bacteria

Test pathogens	**Inhibition zone diameter (mm)**	**MIC (MBC)¶**	**Inhibition zone diameter (mm)**	**MIC (MBC)¶**
	B. velezensis* MBTDLP1		**Chloramphenicol†**	
Methicillin-resistant *Staphylococcus aureus* (ATCC 33592)	$18^{a} \pm 0.02$	15 (15)	$15^{a} \pm 0.20$	6.25 (6.25)
Vibrio parahaemolyticus (ATCC 17802)	$28^{b} \pm 0.25$	7.5 (7.5)	$11^{b} \pm 0.11$	6.25 (10.0)
Vibrio parahaemolyticus (MTCC 451)	$22^{c} \pm 0.10$	7.5 (15)	ND	6.25 (12.5)
Edwardsiella tarda(MTCC 2400)	$17^{a} \pm 0.12$	15 (15)	$8^{c} \pm 0.23$	6.25 (6.25)
Aeromonas caviae (MTCC 646)	$15^{a} \pm 0.06$	15 (15)	$9^{c} \pm 0.32$	6.25 (6.25)
Aeromonas salmonicida (ATCC 27013)	$19^{c} \pm 0.20$	15 (15)	$10^{b} \pm 0.20$	12.5 (12.5)
Streptococcus pyogenes (MTCC 1924)	$21^{c} \pm 0.13$	7.5 (15)	ND	12.5 (12.5)
Escherichia coli (MTCC 443)	$12^{a} \pm 0.05$	15 (15)	$10^{b} \pm 0.23$	6.25 (6.25)

*30 µg/mL of crude extract.

¶The MBC values of candidate bacterium and chloramphenicol were described in parentheses, and expressed as µg/mL.

ND- non-detectable zone of clearance

[a-c] Column-wise values with different superscripts of this type indicate significant difference ($p< 0.05$), which implied for the statistical evaluation of the results. Triplicate values were taken and the variance analyses (ANOVA) were carried out (using Statistical Program for Social Sciences 13.0) for means of all parameters to examine the significance level ($p < 0.05$). Results were expressed as mean ± SD (n = 3).

4.13. Pharmacological properties of the organic extract of *B. velezensis* MBTDLP1

The solvent extracts of *B. velezensis* was assayed in vitro for their antioxidant potential along with their abilities to inhibit various pharmacological targets, pro-inflammatory enzyme (5-LOX), and carbolytic enzymes *α*-amylase and α-glucosidase which were associated with, inflammation, and diabetes respectively. The organic extract of *B. velezensis* exhibited significantly greater antioxidative potential as determined by the scavenging activities of DPPH (IC50 899 µg/mL), ABTS+ (IC50 107 µg/mL) radicals when compared to the standard α- tocopherol against DPPH with 660 µg/mL and 760 µg/mL against ABTS radicals. The ferrous ion-chelating activity was shown by organic extract of *B. velezensis* and positive control EDTA with IC50 values 4203 ± 0.23µg/mL and 18.35 ± 0.99 and µg/mL, respectively. The ethyl acetate extract derived from *B. velezensis* MBTDLP1 exhibited significantly greater inhibition towards inhibit *α*-amylase and α-glucosidase with IC50 120 and 420 µg/mL, respectively when compared to the standard Acarbose (IC90 645 µg/mL). The extracts exhibited considerable inhibition against the pro-inflammatory enzymes, which appeared to play significant functional roles in the metabolic pathway of inflammation. The organic extract from *B. velezensis* found to be selective 5-LOX inhibitors (anti-LOX-5 IC50 17 µg/mL) than commercially available NSAID (ibuprofen 920 µg/mL).

Table 4.5. Pharmacological properties of the organic extract of *B. velezensis* MBTDLP1 assessed by various in-vitro methods.

	Activities IC_{50}(µg/mL)	
Pharmacological properties	***B. velezensis* MBTDLP1**	**Standard**
Antioxidant property		
DPPH radical scavenging	896[a]±0.03	660[b]±0.07
ABTS radical scavenging	107 [a]±0.08	760 [b]±0.02
Ferrous ion chelating	4127 ±0.23	18.35 ±0.99
Anti-inflammatory property		
5-LOX inhibitory activity	17 [a]±0.01	920 [b]± 0.05
Anti- diabetic property		
α-amylase inhibitory activity	120 [a]±0.06	312.44 [b]±0.04
α-glucosidase inhibitory activity	420[a]±0.08	645.08 [b]±0.01

The samples were analyzed in triplicates (n = 3) and expressed as mean ± standard deviation. Means followed by the different superscripts (a,b) within the same row indicate significant difference ($p < 0.05$)

Other notations were as described in the text

[b] The standard antioxidant agent was α-tocopherol, and EDTA for ferrous ion chelating whereas iboprofen and acarbose were used as standard anti-inflammatory and anti-diabetic agents.

4.14. Ex-vivo studies on cancer cell lines

4.14.1. MTT assay

In this study, the test compound was evaluated to analyze the cytotoxicity effect on MCF7 and 3T3L cell lines. The direct microscopic observations of drug treated images of AF-4 after 48 hours of incubation suggest that the test extract is significantly cytotoxic in nature against MCF7 cells and moderately cytotoxic against 3T3-L1 cells. It could be considered that extract is anticancer in nature on MCF7 cells due to its lower IC50 value.

Table 4.6. IC_{50} values of the test compound.

Cell line	MCF7	3T3-L1
IC_{50} (µg/mL)	32.22	140.13

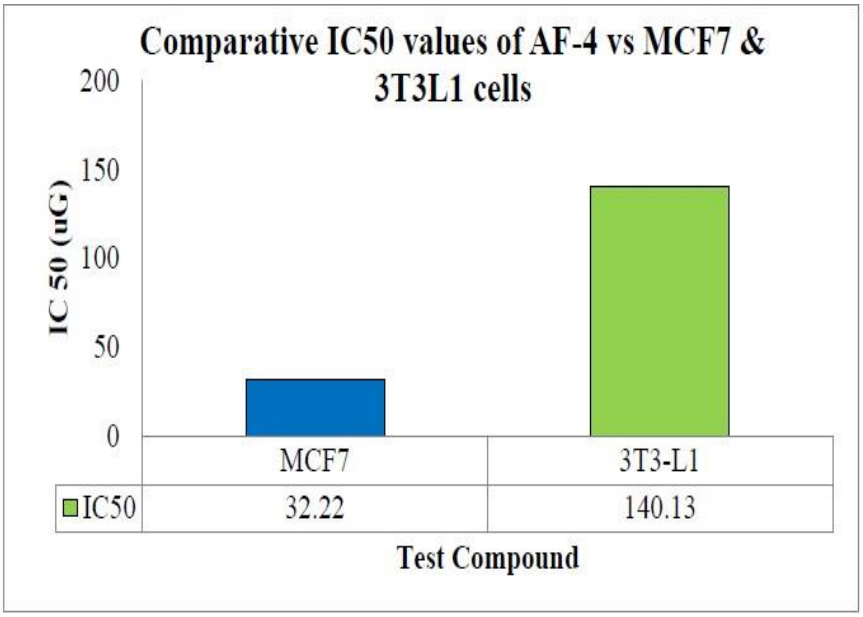


Figure 4.17. IC_{50} values of AF4 on MCF7 and 3T3-L1 after the incubation period of 48hrs.

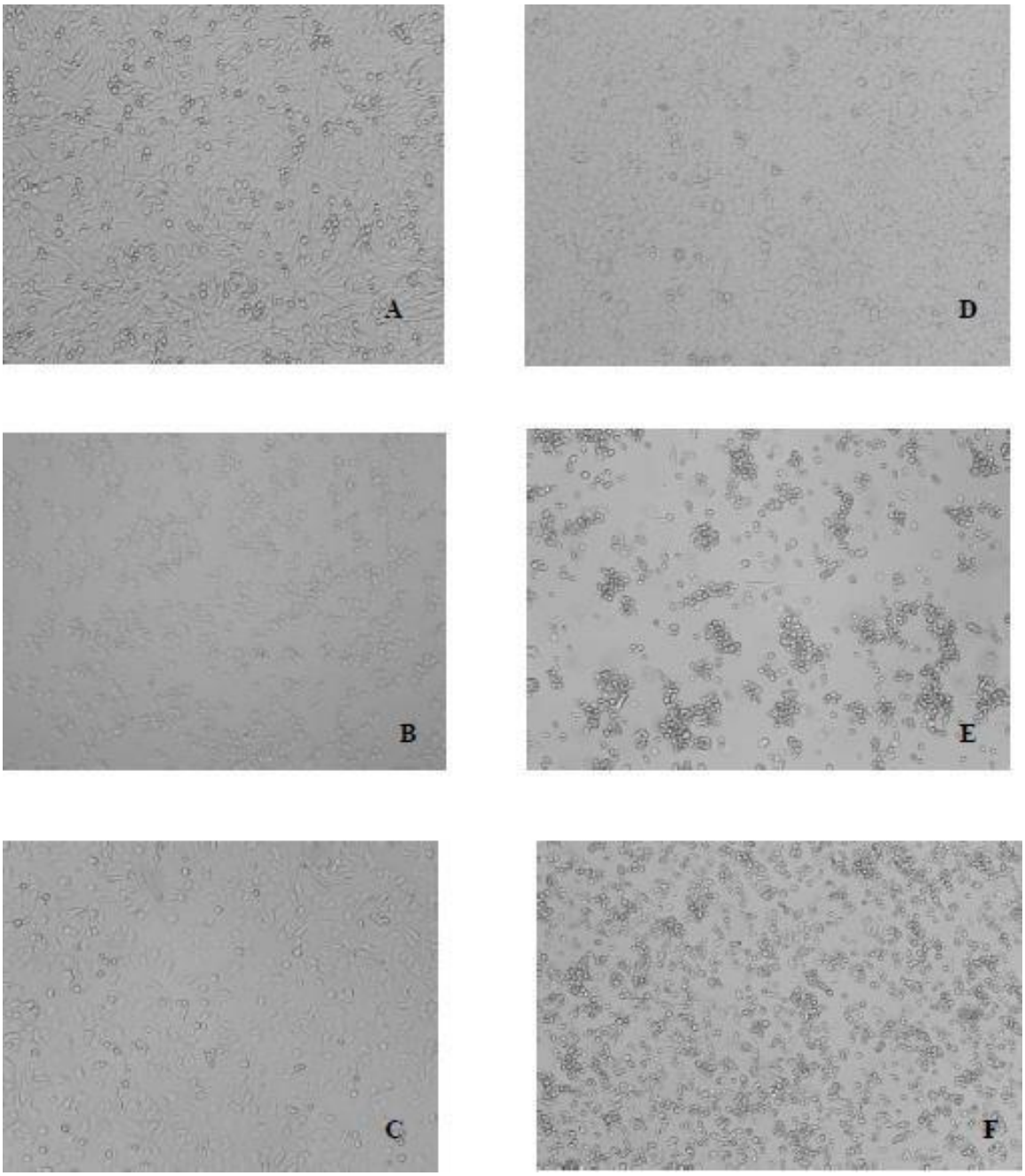


Figure 4.18. Direct microscopic image of the cell lines MCF-7 and 3T3L-1 in response to the test extract to MTT assay for cytotoxicity against cancer cell line and normal cell line. (A) Untreated 3T3L-1 cell line, (B) Standard control on 3T3L-1 cell line, (C) Test extract on 3T3L-1 cell line, (D) Untreated MCF-7 cell line, (E) Standard control on MCF-7 cell line and (F) Test extract on MCF-7 cell line.

4.14.2. NRU assay

The direct microscopic observations of drug treated images of AF-4 after 48 hours of incubation suggests the organic extract is significantly cytotoxic in nature against MCF7 cells on dose dependent manner and moderately cytotoxic against 3T3-L1 cells. Thus it considered that AF-4 as anticancer in nature on MCF7 cells due to its dose response of cell viability.

Table 4.7. Percentage of cell viability of AF4 on MCF7 and 3T3-L1 cell lines.

Concentration(µg/mL)	MCF-7	3T3-L1
Untreated	100	100
Doxorubicin	42.27	56.97
12.5	94.70	98.03
25	72.60	89.47
50	55.03	76.09
100	35.94	64.14
200	19.77	51.42

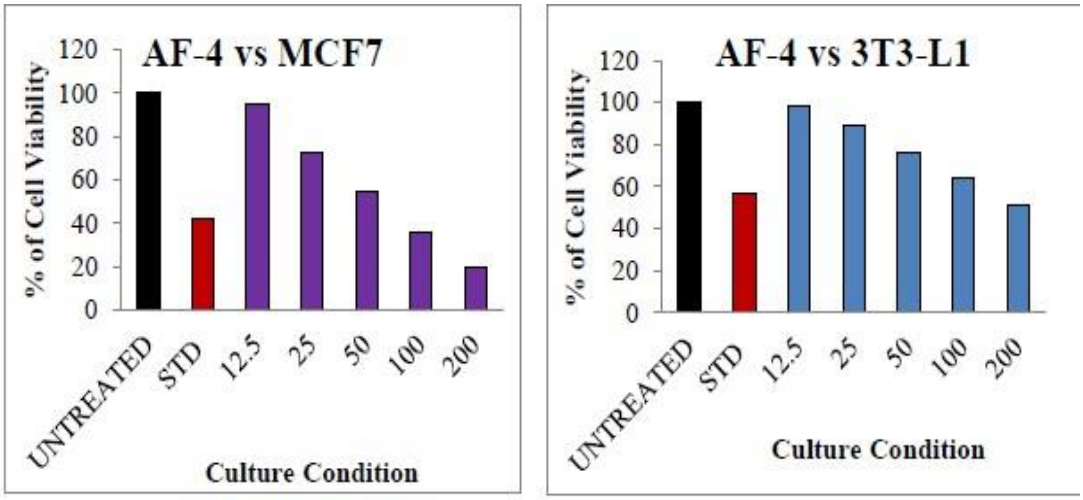


Figure 4.19. Percentage of cell viability of AF4 on MCF7 cell line and 3T3L-1 cell line after the incubation period of 48hrs.

4.14.3. Apoptosis assay

4.14.3.1. Annexin V/PI expression study in MCF7 cell line:

Table 4.8. Table showing the % of cells of undergone Apoptosis in untreated, standard and test compound AF4 (with 1 concentration viz., 32.22µg/mL) treated MCF7 cells.

Quadrant	%Necrotic cells	% Late apoptotic cells	%Viable cells	% Early apoptotic cells
Label	UL	UR	LL	LR
Cell Control	0.03	0.03	99.44	0.50
Std Control	4.13	84.60	2.31	8.96
Test extract (AF-4)	0.57	5.16	12.47	81.80

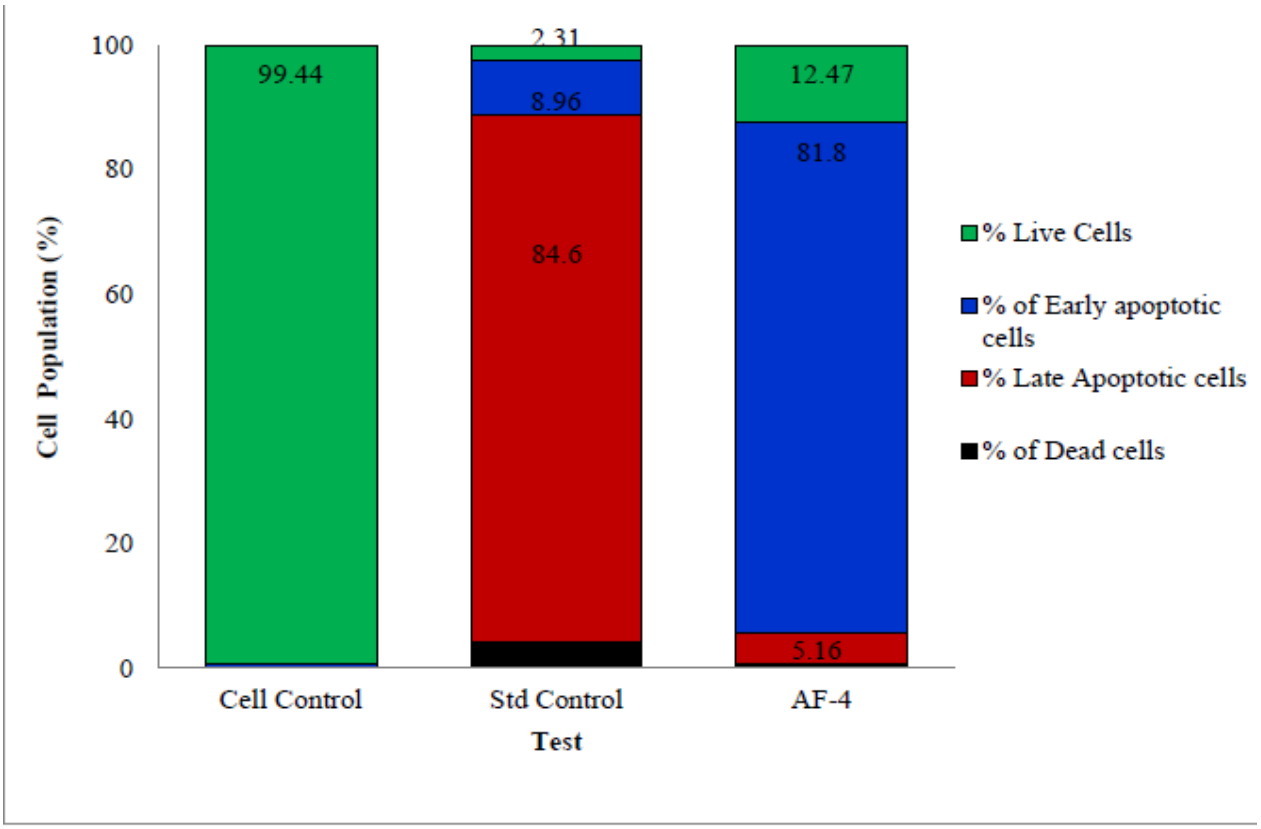


Figure 4.20. Bar graph showing the % of apoptotic and necrotic cells

4.14.3.2. Annexin V/PI expression study in 3T3L1 cell line:

Table 4.9. Table showing the % of cells of undergone Apoptosis in untreated, standard and test compound AF4 (with 1 concentration viz., 140.13µg/mL) treated 3T3L1 cells.

Quadrant	% Necrotic cells	% Late apoptotic cells	% Viable cells	% Early apoptotic cells
Label	UL	UR	LL	LR
Cell Control	0	0.03	99.87	0.1
Std Control	2.82	5.42	45.2	46.56
Test Extract (AF-4)	0	13.58	58.25	28.17

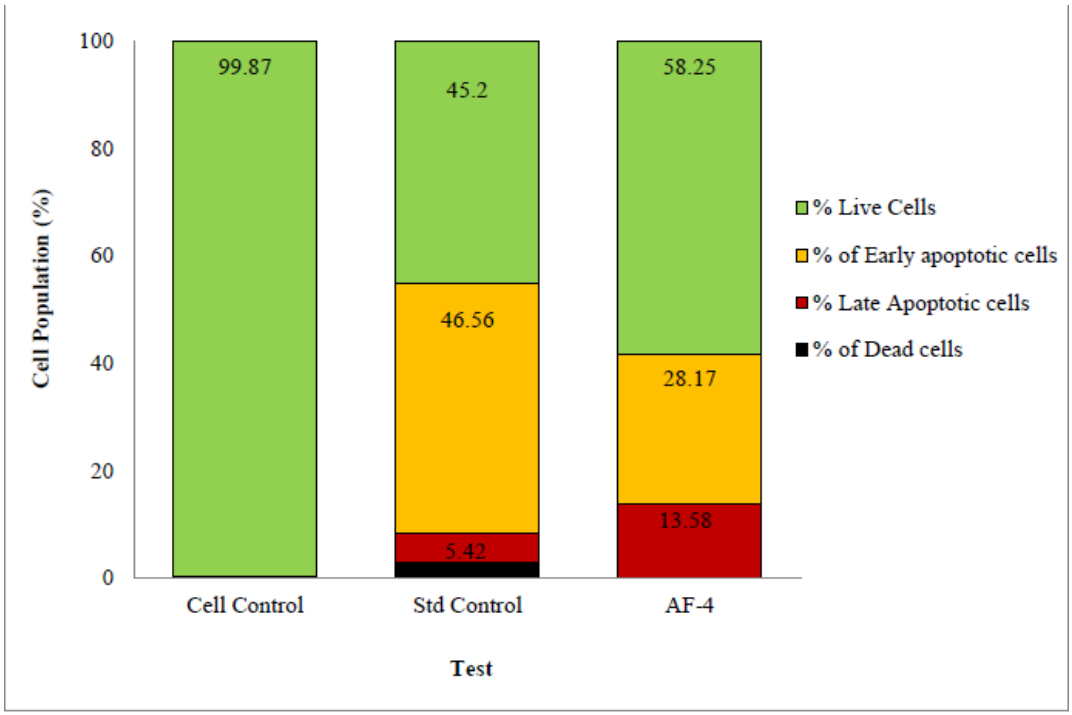


Figure 4.21. Bar graph showing the % of apoptotic and necrotic cells

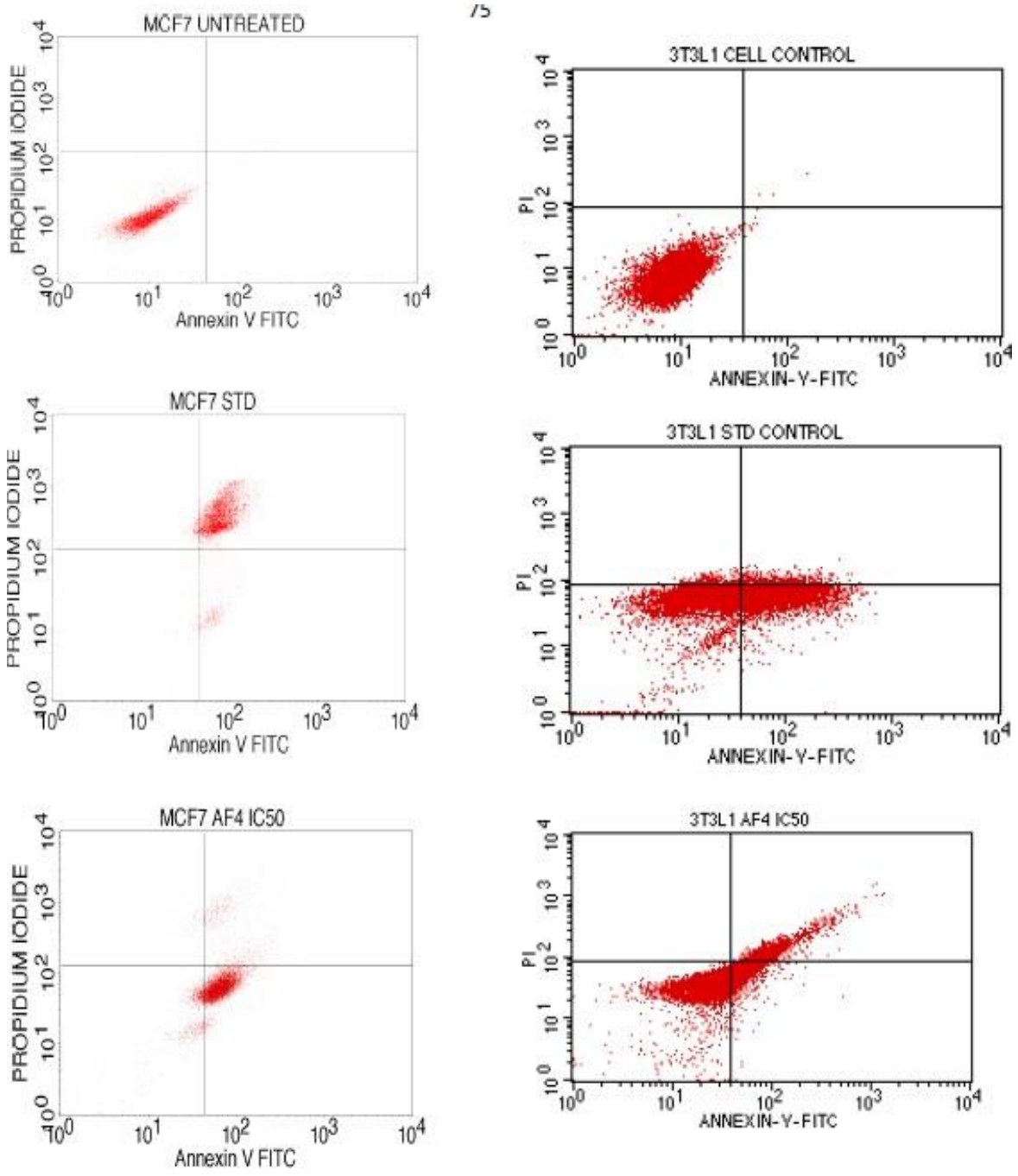


Figure 4.22. Annexin V-FITC-Propidium Iodide expression in MCF-7 and 3T3L-1 cell lines showing proportion of cells undergoing apoptosis. Annexin V-FITC is the primary marker and Propidium Iodide is the secondary marker. (The lower left (LL) quadrant shows the viable cells while lower right (LR) shows early apoptotic cells. The upper right (UR) quadrant gives the late apoptotic cells and upper left (UL) shows necrotic or apoptotic cells).

From the results on ex-vivo studies on cancer cell lines suggest that the test compound is anticancer and cytotoxic against Human Breast cancer cell line MCF-7 and only moderately cytotoxic to Fibroblast cell line (normal cell line) unlike the standard drug, doxorubicin used. The range of cytotoxicity was obtained with IC_{50} of 32.22 ug against MCF-7 and a concentration of 132.42 ug against 3T3L-1 cell line. In apoptosis assay, the test extract was able to induce apoptosis in cancer cell line in which most of the cells move to early apoptosis. While comparing with the standard drug that induces apoptosis to cancer cells and normal cells, the percentage of viable cells is less.

4.15. Spectroscopic analysis of the crude extract

The labelling of protons allied with their characteristic magnetic fields of the functional groups in the ethyl acetate extract of *B. velezensis* MBTDLP1 was recorded by the ^{1}H NMR (Figure 4.17). Aromatic proton signals (δ_H 6.6–8.6) were recorded in the ^{1}H NMR spectral data of the bacterial extract with integral (∑H) of about 17.10. The number of protons at δ_H 2–2.5 were attributed to the acetyl or allylic functionalities, and were recorded to be (∑H 6.92), wherein the proton integrals at the olefnic region (δ_H 4.5–6) was ∑H 3.06. The ethyl acetate extract of *B. velezensis* MBTDLP1 displayed greater proton integral owing to the predominance of electronegative functional groups in the downfield region of the NMR spectrum.

Table 4.10. Proton integral table of crude extract

Proton integral (Σ H) at specified chemical shift (δ)

Types of protons (Chemical shift, δ in ppm)	**δ 0.5-2.00[a]**	**δ 2.10-2.50[b]**	**δ 2.6-3.5[c]**	**δ 3.6-4.5[d]**	**δ 4.6-6.5[e]**	**δ 6.6-8.5[f]**
***B. velezensis* MBTDLP1**	75.93	6.92	5.94	12.05	3.06	17.10

[a]Saturated hydrocarbons, [b]Alkyl alkanoates/allylic/acetyl groups (RCH_2C (=O) OR_1)/ (CH_2=$CHCH_3$)/(RC(=O) CH_3), [c](OCH_3)/(RCH_2-X)/ (RCH_2OH), [d]Polysaccharides, [e]Alkanoates/olefinic (RCH_2C(=O)-OCH_3)/ (RCH=CHR_1), [f]Aromatic proton (Ar-H).

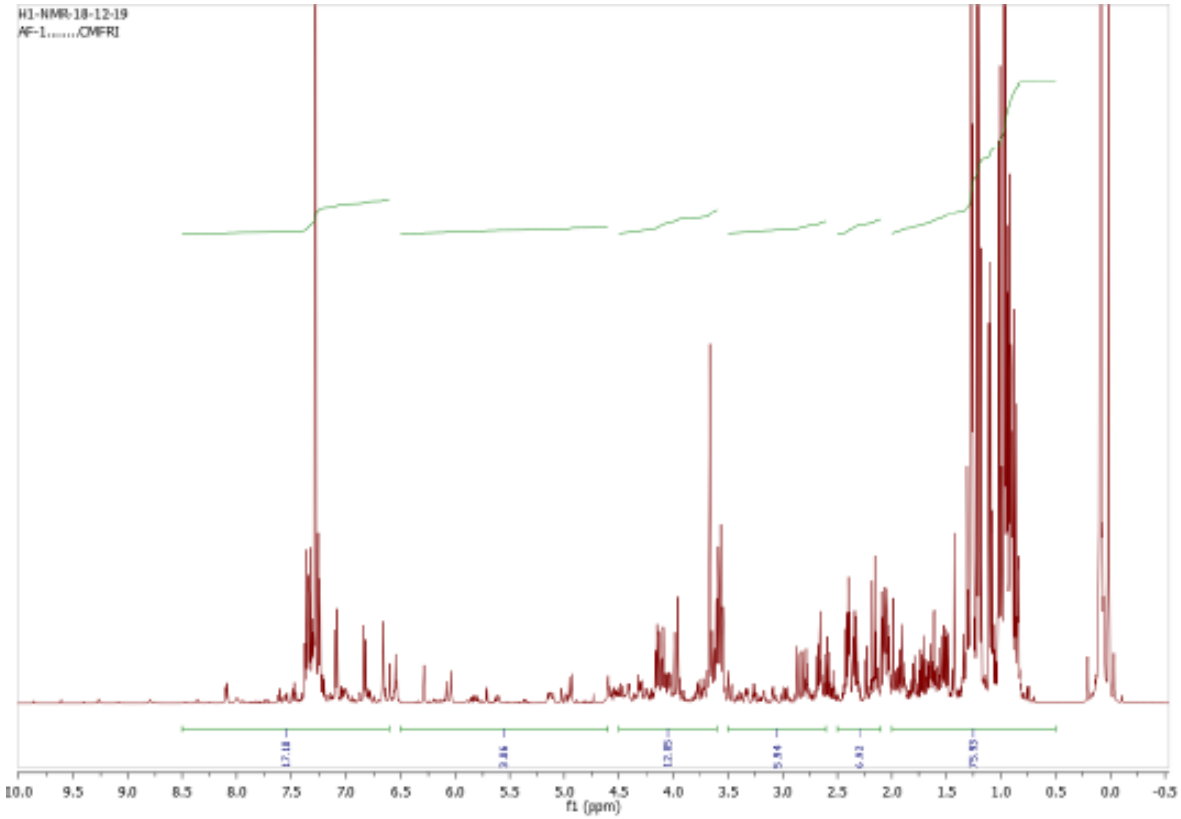


Figure 4.23. ^{1}H NMR fingerprinting of the organic extract of the bacterium, *B. velezensis* MBTDLP1

DISCUSSION

5. DISCUSSION

The association of marine eukaryote with its symbiont microorganisms has been the foundation for exploring the bioactive compounds that extends its applications to multiple fields of interest. The studies revolving the marine free-living and symbiotic bacteria had been propagated since the 1950s after the discovery of their ability to produce antimicrobial metabolites, even though their occurrences were confirmed only in late 1990s (Vijayalakshmi *et al*. 2008). These adaptations in response to survive the increasing competition have opened up the hub for the ominous products that could act against many pathogens. Since last decades, several formulations from microbial sources were in clinical use for the treatment of cancer and infectious diseases, and in organ transplantation as immune suppressors (National Research Council 1999). However, studies and literature involving macroalgae and associated bacteria were limited until last decade (Vijayalakshmi *et al*. 2008). The co-existence of diverse microbes on the same surface enables them for antibiotics production, bacteriocins, siderophores, lysosomes, proteases and pH alteration by producing organic acids (Soria-Mercado *et al*. 2012).

At a recent time, heterotrophic bacterial communities associated with marine algae have attracted attention of natural product chemists, owing to their potential to biosynthesize structurally divergent class of compounds with potential pharmacological significance (Winter *et al*. 2016). Microbial associations harboring promising pharmaceutical properties were reported from the internal tissues of invertebrates and marine plant surfaces (Webster and Taylor 2012). The marine algae was mainly reported to be in association with the bacterial groups, such as *Firmicutes, Bacteroidetes* and *Gamma-proteobacteria* (Wiese *et al*. 2009). They are also reported to possess They have also been demonstrated to possess varied properties of potential therapeutic applications, such as anti-diabetic adjuvant, anti-inflammatory formulations, and antioxidant activities (Tang *et al*. 2010). These microbes were found to produce varied secondary metabolites, such as polyketides, non-ribosomal proteins and bacteriocins that have potential therapeutic applications (Sun *et al*. 2019; Kizhakkekalam *et al*. 2020; Chakraborty *et al*. 2020).

In this study, the intertidal macroalgae belonging to the classes Rhodophyceae and Phaeophyceae were evaluated for the presence of associated heterotrophic bacteria. A total of 8 marine algae samples, namely *S. wightii, G. edulis, T. ornata, P. pavonica, S. tenerrimum, S. marginatum, L. papillosa* and *G. salicornia* were collected from the Southeast coast of Peninsular India. From these samples, a total of 40 bacterial isolates was obtained, in which 20 % of the isolates was contributed by *S. wightii*, followed by *T. ornata, P. pavonica, S. tenerrimum and S. marginatum*, each contributed 12.5 % of isolates each. The marine algae *L. papillosa* and *G. salicornia* contributed a total of 10 % of the isolates and *G. edulis*, with a share of 4 % isolates. The overall distribution of microbial diversity in the present study has been represented as a pie chart. Among the bacterial isolates, the majority of the isolates belonged to the phyla *Gammaproteobacteria* (62 %) from which 18 % of bioactive isolates were obtained. The following 38 % of isolates represented Firmicutes from which the majority of the bioactive isolates was retrieved. The distribution of bioactive isolates associated with marine algae is represented as percentage contribution. Stincone and Brandelli (2020) reported a significant share of antimicrobial compounds from the marine algae associated bacteria. Moreover, the isolates from brown and red macroalgae were reported to possess antibacterial activities against several pathogens including those, which were drug resistant, such as MRSA (Thilakan *et al*. 2016, Rasheed *et al*. 2020, Kizhakkekalam *et al*. 2020).

The isolates from the marine macroalgae were screened to assess their antagonist activities, primarily by spot-over-lawn assay. Despite selecting the isolates displaying inhibition against any one of the test pathogens, the most active isolates, which showed broad-spectrum of inhibitory activity against the pathogens of clinical significance, were selected for further studies. The strains LP1 and LP2, which were characterized as *Bacillus* sp, showed significant antagonistic property against various test pathogens of human and food borne diseases, including MRSA, with a zone of inhibition of 36 and 32 mm, respectively. The methicillin-resistant S. aureus (MRSA) is said to be destructively pathogenic, causing serious infections. The acquired resistance of MRSA against various drugs has pushed the scientific world to find new alternatives, which directed the way to exploit the potential of marine microbial metabolites (Archer *et al*. 2011). Notably, the metabolic and physiological capacities allow the heterotrophic bacteria associated with the marine organisms to survive in the extreme conditions that evolve them for the production of novel bioactive compounds with pharmacological potential, which

were not yet discovered in the terrestrial ecosystems (Faulkner, 2002). The strains MBTDLP1 and MBTDLP2 isolated from the red algae *L. papillosa* were categorized under the genus *Bacillus* by biochemical characterization. The Gram-positive, rod shaped isolates showed positive catalase reaction followed by oxygen requirement for their growth with spore morphology and carbohydrate utilization converging into the *Bacillus* sp. Positive results corresponding to starch hydrolysis, motility, acetoin production, citrate utilization, nitrate reduction and arginine utilization also added to the observations. Further, the 16S rRNA sequence analysis revealed the isolates to be *B. velezensis* MBTDLP1 and B. altitudinis MBTDLP2 belonging to the family Firmicutes. *B. velezensis* was suggested as a synonym of *B. amyloliquefaciens* (Cristina *et al*. 2005). *B. velezensis* FZB42 was reported as the Gram-positive model strain for plant growth and biocontrol (Fan *et al*. 2018). *B. velezensis* has already been reported to be effective against aquatic pathogen *Aeromonas veronii*, an aquatic pathogen that causes hemorrhagic septicemia in both humans and animals (Zhang *et al*. 2019). Also Ma *et al*. 2020 reported the application of marine derived *Bacillus velezensis* 11-5 as a fungicide agent to *Magnaporthe oryzae* in rice. Thus, *B. velezensis* have possessed the properties of a plant growth promoter and biocontrol agent against phytopathogens, aquatic pathogens and human pathogens. Generally, the Firmicutes comprising spore-forming genera allows it to survive for long periods and adopt better survival strategies even under severe environmental conditions (Núñez-Montero *et al*. 2018). Among the Firmicutes group, *Bacillus* species were reported to be dominantly present on the surface of diverse marine algae, and were acknowledged as renowned sources of bioactive compounds with therapeutic significance (Goecke *et al*. 2010). Many diverse molecular bioactives from the genus *Bacillus* have the potential to embark efficient tactics to fight animal, aquatic, human, and phytopathogens in bio-rational ways (Mondol *et al*. 2013). Marine *Bacillus* species were accounted for to produce promising bioactive metabolites, such as polyketides, fatty acids, bacteriocins, and other unusual compounds of potential clinical significance (Goecke *et al*. 2010).

Non-ribosomal peptide synthetase (*nrps*) catalyzes synthesis of oligopeptides and polyketide synthase (*pks*), which catalyze the elongation of polyketides, are the main enzymes for biosynthesis of secondary metabolites and some as a result of their hybrid functionalities (Wang *et al.,* 2014). Some secondary metabolites are encoded by a blend of *nrps* and *pks genes* (Brakhage 2013). Herein, the *pks* amplicon of *B. velezensis* MBTDLP1 (MT394492) revealed

resemblance to Type-I pks of *B. velezensis* in the BLAST similarity search. Marine macroalga-associated heterotrophic *B. subtilis* with *pks-1* gene, showed a new variant of polyketide furanoterpenoids with potential activity against food-borne pathogens (Chakraborty *et al*. 2017). A rare chemistry of polyketide-spanned elansolid-type macrolides showing potential growth inhibition on drug-resistant bacteria were isolated from *B. amyloliquefaciens* associated with H. valentiae (Kizhakkekalam *et al*. 2020). The genome of heterotrophic *B. amyloliquefaciens* was also reported to synthesize different bioactive compounds that are in fact result of biosynthetic gene clusters involved (Kizhakkekalam *et al*. 2020; Chakraborty *et al*. 2020).

Most of the bacteria secrete strong iron-chelating molecules, such as siderophores for scavenging iron from their surroundings that are taken up by specific receptors (Kramer *et al* 2020), that can establish a competition between bacterial species for iron acquisition. This ability of bacteria can contribute to the competitive characteristics that enable their antagonist activity against the pathogenic bacteria. An earlier report of literature found that the siderophores produced by *B. cereus* was inhibitory for the growth of *A. hydrophila* (Lalloo et al. 2010). Siderophores are typically synthetized by nrps or pks domains that work in coordination with nrps modules (Kramer *et al*. 2020). Bacterial isolates associated with the marine algae, *Ulva lactuca* showed orange halo on CAS agar plates indicating the siderophore production in which *B. subtilis* was present as one of the isolates, thus helping the algae to survive the iron-limiting conditions (Naik *et al*. 2019). Although Gram-positive bacteria such as *Bacillus*, *Staphylococcus* and *Streptomyces* also possessed siderophore-mediated iron transport systems, this metabolite had been extensively discussed for Gram-negative bacteria until their discovery in Gram-positive bacteria (Jenifer *et al*. 2015). *B. subtilis* was reported to be a potent biocontrol agent with siderophore excretion (Hu and Xu 2011). The present study reported the production of siderophore in the isolate of *B. velezensis* MBTDLP1 on the CAS agar plates.

The organic extract of the selected isolate, *B. velezensis* MBTDLP1 exhibited significant antibacterial activities against the test pathogenic bacteria in disc-diffusion assay. The zone of inhibition showed activity against the test pathogens varied from 13-25 mm, even against the drug-resistant strain, MRSA. The positive control of chloramphenicol showed a comparatively lesser zone of inhibition against these pathogens. The MIC of the organic extract was found to be 7.5 µg/mLl against test pathogens such as *V. parahemolyticus* and *S. pyogenes*, and the standard

chloramphenicol showed a MIC of 6.25 µg/mL against MRSA, *V.parahemolyticus* and 12.5 µg/mL against *S. pyogenes* . Karthick and Mohanraju (2018) isolated a total of six isolates from 8 marine macroalgae from which the red algae, *Gracilaria corticata* harbored bioactive *Bacillus* sp. with significant antibacterial activity against *K. pneumoniae* and *S. aureus*.

In the present study, the disc diffusion assay showed lesser zone of clearance when compared with preliminary screening of antibacterial activity. The probable reason behind the results might be the difficulty of the extract in diffusing from the discs to the agar media as the compounds might be strongly adhered to the cellulose discs used. The standard filter paper (Whatman, 6 mm) used in the antibiotic disc assays is composed of cellulose. The surface of the disc is rendered hydrophilic because of the free hydroxyl groups existing on each glucose residues (Braithwaite and Smith, 1990). Thus, if heterotrophic marine bacterium produces compounds that are cationic, then the compounds can stay adhered to the disc surface, thus not diffusing into medium. Thus, even if the bacteria produces compounds that are antibacterial in nature, it does not need to be expressed in disc diffusion assay (Burgess *et al.* 1999). Interestingly, the proton NMR integral was higher at the region of δH 3.6–4.5 (∑H 12.05) in the organic extract of the studied bacterium, which leads to the conclusion that the bioactive compounds present in the extract might be highly polar in nature. However, the MIC of the organic extract (7.5 µg/mL) was found to be comparable with that of the standard antibiotics (6.25 µg/mL), against most of the pathogens. Thus, considering the preliminary screening and micro dilution method, it could be inferred that the bacterial extract possesses strong inhibition against the bacterial pathogens, and lesser zones of inhibition in disc diffusion assay might be the result from its greater polarity.

The pharmacological evaluation of the extract revealed the potential of the organic extract as antioxidant, anti-inflammatory, anti-diabetic, anti-bacterial and anti-cancer agent. Cao *et al.* 2020 reported that exopolysaccharides from *B. velezensis* possessed strong anti-oxidant potential against DPPH, ABTS, and O_2^- radicals. Here, the radical scavenging activity varied as for DPPH radical 896 µg/mL of concentration marked for IC_{50} and ABTS radical scavenging was done with IC_{50} value of 107 µg/mL. The ABTS assay detect antioxidant capacity of hydrophobic and hydrophilic extracts while DPPH predicts antioxidant capacity for hydrophobic antioxidants, that serves to the higher values obtained in ABTS compared to DPPH scavenging activity (Al-Zereini 2014). The ferrous ion chelating ability was assessed to be relatively lower when compared to

established metal ion chelators. The IC_{50} value was determined to be 4.2 mg/mL. Due to electron transport in mitochondria and related metabolic responses, ROS (reactive oxygen species) is produced as a by-product of these reactions. Furthermore, metal catalyzed oxidative reactions lead to the formation of (ROS). The formation of free radicals can be achieved by the Fe^{2+} ion by the gain or loss of electrons (Adjimani *et al*. 2015). Thus, chelating agents that can collectively chelate these ions can reduce the formation of ROS. The ferrous ion chelating activity contributes to be an effective antioxidant property for iron-related pathways (Sudan *et al*. 2014). The ferrous ion chelating assay performed to assess the ferrous ion chelating activity of the bacterial organic extract unveiled moderate chelating activity with IC_{50} of 4.2 µg/ mL. Hamidi *et al*. (2019) reviewed marine-derived *Bacillus amyloliquefaciens* 3MS 2017 produced exopolysaccharides that exhibited 64 % ferrous ion chelation with a concentration of 100 µg/mL. Combining the observations from the study, the production of siderophore molecules together with the moderate ferrous ion chelation recognized its potential to scavenge iron from the surrounding environment, and thus creating a competition to the pathogenic bacteria along with reducing the ROS accumulation in the body. Moreover, siderophore conjugated antibiotics were reported to be easily transported into the cells of Gram-negative bacteria by exploiting their iron acquisition pathways (Kong *et al*. 2019). The biocontrol mechanism is dependent on the competition between the transferrin molecules by pathogens and the siderophore molecules produced by bioactive microorganism in forming complexes with iron, and the siderophore always have the upper hand because of its much higher iron stability constants as reported by previously (Gram et al. 1999).

The organic extract exerted an anti-inflammatory activity against 5-LOX with IC_{50} value of 17 µg/mL and inhibition of carbolytic enzymes, α-amylase and α-glucosidase was marked to be 120 µg/mL and 420 µg/mL respectively possessing anti-diabetic activity. The secondary metabolites of marine bacterium *Streptomyces sp*. VITASP exhibited antibacterial activity at a concentration of 0.5mg/mL against two Gram- positive and Gram- negative bacteria. The antioxidant potential of the crude extract exhibited strong reducing power activity with 93±0.05% inhibition at a concentration of 0.5 mg/mL. The crude extract possessed anti-inflammatory and anti-diabetic properties at a concentration of 0.5 mg/mL. The cytotoxic effect with an IC_{50} with a value of 500 µg/mL was found on HeLa cell lines (Thosar *et al*. 2020). A major amount of marine bacteria under wide range of bacterial phyla such as *Firmicutes, Actinobacteria, Proteobacteria* and

Bacteroidetes, were found to produce beta glucosidase inhibitors for use as anti-diabetics, anti-obesity and antitumor compounds (Pandey *et al*. 2013). Two marine heterotrophic bacteria, *B. amyloliquefaciens* MTCC 12716 and *S. algae* MTCC 12715 associated with the algae *Hypnea valentiae* reported with exceptional pharmacological potential keeping high profile in comparison to the available standards (Kizhakkekalam and Chakraborty 2020). The contribution of the Firmicutes lined *Bacillus sp*. marks the resource for most potential compounds among the products deciphered so far. Two polyketides were retrieved from a marine bacterium *Bacillus licheniformis* from a sediment core sample collected on the southern Iodo reef, Republic of Korea. The compounds ieodoglucomides A and B of polyketide class of compounds showed in vitro antimicrobial activity against Gram-positive and Gram-negative pathogenic bacteria (MIC = 8–32 µg/mL). Moreover, the antitumor properties of the compound ieodoglucomide B exhibited cytotoxic activity against gastric cancer cell line (GI_{50} = 17.78 µg/mL) and lung cancer cell line (GI_{50} = 25.18 µg/mL) (Tareq *et al*. 2012).

Reports of anti-bacterial and cytotoxic activity of the extracts and fragments of marine bacterium of *Streptomyces* genus has been explored on breast cancer cell line MCF-7 with IC_{50} values 83.65 µg/ mL against MCF-7and 31.88 µg/ mL and 68.35 µg/ mL against ductal carcinoma cell lines ZR-75-30 and T47D (Cartuche *et al*. 2015). Here, the organic extract imparted cytotoxicity against the cancer cells (MCF-7) with concentration of IC_{50} values 32.33 µg/ mL and much lesser cytotoxicity against normal cells 3T3L-1 requiring a higher concentration of IC_{50} of 140.32 µg/ mL. The Annexin V-FITC/PI studies for apoptosis assay gave the quantitative measure of viable, apoptotic and necrotic cells. The cell lines treated with IC_{50} concentration of the organic extract showed much lower viability in case of breast cancer cells showing up to 81 % cells at early apoptosis stage while it maintained cell viability of normal cells almost to 60 %. Induction of apoptosis was reported in lung carcinoma cell line A547 cell line with 60 % of apoptotic cells by the cyclic lipopeptides from marine algae associated *Bacillus atrophaeus* AKLSR1 (Routhu *et al.* 2018). Even though increased interest and studies are carried out in marine niches, they are largely unexplored for anti-cancer compounds. Many isolated compounds with anticancer properties from marine origin are identified and characterized. Some are under clinical trials for human consumption and administration (Wali *et al*. 2019). The organic extract can be explored further and structurally characterized for the detailed studies as a pharmacological agent. Due to the high pharmacological potential of the marine microbial derived products and the feasibility

of their production in an eco-friendly way, these compounds have occupied an important role in the therapeutic intervention of many complex diseases and still today represent a crucial alternative to treat different immunological, inflammatory and malignant conditions (Riera-Romo *et al*. 2020).

Thus the look and need for naturally occurring bioactive compounds with therapeutic potential has always been on the top list since decades. With a massive reservoir of the diverse ecosystems within and biodiversity, marine habitats will always be a continual source of potential compounds. The flora and fauna along with their associated microbial communities will be the unraveling pool of natural products from marine origin. The bacterium, *B. velezensis* MBTDLP1, a simple unicellular inhabitant of a marine alga has turned out to be a complex therapeutic agent with anti-bacterial, anti-oxidant, anti-inflammatory, anti-diabetic and anti-cancer activities.

SUMMARY

6. SUMMARY

The need for the resources to meet the pharmacological requirement in this era of increasing population is a mandatory. Thus, a marine ecosystem that harbors diversity within biodiversity gives the key to unlock the solution to this scenario (Pandey 2019). Marine algae associated heterotrophic bacteria have already found their way to the limelight of marine sciences with their bioactive compounds. Several bioactive bacteria have been isolated and evaluated worldwide and in the country for their application in pharmaceutical science. Bioprospecting of marine microbes has recognized many astounding landmarks in pharmacology, drug designing, and therapeutics. Marine *Bacillus* are known to possess metabolites with diversified functionalities such as macrolactones, lipopeptides, non-ribosomal peptides, polypeptides, polyketides and coumarins highlighting a wide array of diverse pharmacophores, ranging from antimicrobial, antidiabetic, anti-inflammatory, antioxidant and anticancer in nature, heavy metal detoxification, carotenoids production and even biocontrol agents and biopesticides (Cherian *et al*. 2019).

The proposed work was conducted at the Marine Biotechnology Division, ICAR-CMFRI, Kochi.

In this present study, heterotrophic bacteria associated with marine algae was isolated, screened, characterized, and subjected to bioprospecting and finally pharmacological evaluation. A total of 40 bacteria were isolated and screened for their antibacterial property. Among 40 isolates, 7 showed activity from which the two most active isolates that showed collective antagonist activity against the test pathogens were selected for further studies. The two isolates imparted anti-bacterial activity against multi-drug resistant, Methicillin resistant *Staphylococcus aureus* (MRSA), *Vibrio parahemolyticus, Streptococcus pyogenes, Yersinia enterolytica* with inhibition zone >20 mm of diameter. The isolates were selected based on their strong anti-infective property and further subjected to anti-oxidant property that showed positive scavenging activity with a zone of inhibition 15 mm.

The isolates were then identified through morphological, biochemical and molecular characterization. The white, undulated, Gram-positive colonies of the isolates gave positive results in biochemical characterization that confined the isolates to *Bacillus* genus and similar to *Bacillus subtilis*. The 16S rRNA sequencing of the DNA of the isolates characterized them as

Bacillus velezensis and *Bacillus altitudinis* which were later named as *Bacillus velezensis* MBTDLP1 and *Bacillus altitudinis* MBTDLP2. Screening of the functional genes was performed to filter out Type-I polyketide producers by the expression of polyketide synthase genes. Positive amplification was obtained for *B. velezensis* MBTDLP1 with an amplicon of 700 bp while no amplification was given by the other isolate. Thus, for further bioprospecting and pharmacological evaluation studies, the most active, polyketide producing *B. velezensis* was selected.

The bacteria gained stationary phase at 68-72 hours and extraction was carried out based on the observation. The bacterium was grown over nutrient agar and the secondary metabolites were exhaustively extracted using ethyl acetate solvent at the stationary phase. The organic extract was collected (5g), concentrated, dried in nitrogen gas and stored in vacuum. Thin layer chromatography was performed in various solvents to deduce the type of compounds present in the extract. The TLC plates showed good separation of spots in 100 % ethyl acetate solvent. The TLC plates were then run in UV light (365 nm and 254 nm), iodine, alcoholic KOH, sulphuric acid-formaldehyde giving the assumption for the presence of compounds such as anthrones,

The pharmacological evaluation of the organic extract was assessed by their anti-bacterial activity, antioxidant activity, anti-inflammatory activity, anti-diabetic activity and anticancer activity. The extracellular metabolites of *B. velezensis* MBTDLP1 were extracted using an organic solvent, here by ethyl acetate to yield the organic extract (5 g). The antibacterial activity of the organic concentrate was assessed by disc diffusion assay and MIC (by micro dilution method). The antagonist activity showed 28 mm of zone against *V. parahaemolyticus* ATCC 451, and 18 mm against MRSA ATCC 33952, in descending order. It was observed that the MIC of the organic extract of *B. velezensis* MBTDLP1 was 7.5 µg/ mL against *V. parahaemolyticus and S. pyogenes* and 15 µg/ mL against MRSA.

The solvent extracts of *B. velezensis* was evaluated in vitro for their antioxidant potential along with their abilities to impart inhibition of various pharmacological targets, α-amylase and α-glucosidase, pro-inflammatory enzyme (5-LOX), which were associated with diabetes, and inflammation respectively. The organic extract of *B. velezensis* exhibited significantly greater anti-oxidative potential against DPPH (IC_{50} 896 µg/ mL) and ABTS+ (IC_{50} 107 µg/ mL) radical scavenging when compared to α-tocopherol (IC_{50} 660-760 µg/ mL). The organic extract from *B.*

velezensis exhibited inhibition towards inhibition of *α*-amylase and α-glucosidase with IC_{50} 120 and 420 µg/ mL, respectively when compared to the standard Acarbose (IC_{90} 645 µg/ mL). The extracts presented extensive inhibition against the pro-inflammatory enzymes, which appeared to play substantial functional roles in the metabolic pathway of inflammation. The organic extract was found to be selective 5-LOX inhibitors (anti-LOX-56 IC_{50} 17 µg/ mL) than commercially available NSAID (ibuprofen anti-LOX–5 IC_{50} 920 µg/ mL).

The determination of anti-cancer property was carried out by cytotoxicity assays and apoptosis studies on MCF-7 (Human breast cancer cell line) and 3T3L-1 (fibroblast normal cell line) with an anti-cancer drug, doxorubicin as drug/standard control. From the MTT assay, the organic extract exerted cytotoxicity to MCF-7 cells with IC_{50} concentration 32.22 µg/ mL and 140.13 µg/ mL against 3T3L-1 cells. In NRU assay, the extract imparted cytotoxicity in a dose dependent manner at a concentration of 200 µg/ mL restricting cell viability to 19 % in MCF-7 and maintained 51 % viability in 3T3L-1 cells. The induction of apoptosis was studied with IC_{50} concentration (32.22 µg/ mL) in which the extract induced early apoptosis to 81 % of the cancer cells and maintained 60 % of viability in 3T3L-1 cells.

Thus, with the strong anti-bacterial activity coupled with the therapeutic potential, the selected isolate can be explored further to be characterized and developed as a potential pharmacophore agent.

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6. REFERENCES

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DEVELOPMENT AND PHARMACOLOGICAL EVALUATION OF SMALL MOLECULAR BIOACTIVES FROM MARINE-ALGAE ASSOCIATED HETEROTROPHIC BACTERIA

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ABSTRACT

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Increased incidences of microbial resistance and the development of drug-resistant pathogens have triggered an urge amongst researchers focusing on the discovery of potential anti-infective compounds. The present study conducted at Marine Biotechnology Division, ICAR-CMFRI, Kochi, highlights the cultivable diversity and bioactivities of heterotrophic bacteria associated with the intertidal macroalgae of Southeast coast of India. Among the 40 bacteria isolated, the majority of the isolates represented *Gamma-proteobacteria* (62 %) and the following 38 % resided in the phyla *Firmicutes*. The isolates were then screened for antibacterial activities against a wide spectrum of pathogens including methicillin-resistant *Staphylococcus aureus* by spot on lawn assay. Further, one of the most active strains belonging to the phyla *Firmicutes* isolated from Rhodophyceae macroalga, *Laurencia papillosa*, with a zone of inhibition ≥ 35 mm, was selected for bioprospecting studies. It was characterized as *Bacillus velezensis* MBTDLP1 (MT122835), based on integrated phenotypic and genotypic analysis. *Type-I pks* gene (MT394492) of 700 bp also could be amplified from the candidate bacterium. The bacterium exhibited siderophore production in CAS agar screening. The bacteria showed significant susceptibility to the commercially available antibiotics thus neglecting the chances of pathogenicity. The organic extract of the candidate bacterium was also found to display ferrous ion chelating capacity (IC_{50} 4.2 mg/mL). The heterotrophic bacterium *B. velezensis* MBTDLP1 exhibited promising anti-infective properties against test pathogens which included multidrug- resistant pathogen methicillin-resistant *S.aureus* (MIC of 15 µg/mL). The organic extract showed antibacterial activity (MIC 7.5-15 µg/mL), antioxidant property with IC_{50} values from 0.1-0.9 mg/mL against ABTS and DPPH radicals, anti-inflammatory activity with IC_{50} 0.01 mg/mL against 5-LOX, anti-diabetic property with IC_{50} 0.1-0.4 mg/mL in correspondence to α-amylase and α-glucosidase. The organic extract also showed significant anti-cancer activity against Human breast cancer cells with IC_{50} 0.032 mg/mL and less cytotoxicity to normal cells. Significant antibacterial activity against drug resistant bacteria together with considerable pharmacological activities combined with the presence of genes coding for bioactive secondary metabolites revealed that this marine symbiotic bacterium could be used against the dilemma.