



## Assessment of antibacterial efficacy of the methanolic extract of *Bacillus vietnamensis* PBChS1 isolated from marine sediments of Chidiyatapu coast, South Andaman

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Marine bacteria are of extensive significance as new ‘budding springs’ of a vast number of organic active artefacts. Till now, only a fraction of microorganisms have been scrutinized for bioactive metabolites, yet a colossal sum of these metabolites with unique structural drafts remains untouched and unexploited. In this respect, the species of *Bacillus vietnamensis* was isolated from the marine sediments of Chidiyatapu coast, South Andaman and characterized by both phenotypic and genotypic methods. The anti-oxidative activities of the methanolic extract were determined by the estimation of TPC (Total Phenolic Content), reducing power, free radical scavenging assay and ferric thiocyanate method. Antibacterial activity of the methanolic extract was analyzed by an agar well diffusion assay confirming the presence of active metabolites of various functional groups by FT-IR, thereby warranting a ‘combinatorial approach’ for applicative pharmacological settings.

[**Keywords:** Antibacterial activity, Antioxidant assay, *Bacillus vietnamensis*, FT-IR, Marine sediment, Secondary metabolite]

### Introduction

The term “metabolome” (as proposed in 1997) refers to the metabolites (both primary and secondary) synthesized by a microorganism<sup>1,2</sup>, indicative of its bio-function related to its phenotypic sketches<sup>3</sup>. Metabolites are classified as chemical species of small molecular weight [ $< 1000$  daltons (Da)] exhibiting a significant part in microbial metabolism, chemical translation and transformation with its three defined and discrete matrices of configurations: intra-cellular, extracellular and medium headspace<sup>4</sup>. They are classified into three types based on their solvent solubility as water-soluble, water-insoluble and volatile types<sup>5</sup>, accrediting their diversities among assorted metabolites corresponding to higher factors at atomic rank rather than at molecular levels<sup>1</sup>. The schemes of metabolic conduits compounding precursors of primary metabolism are coupled with the processes of production (anabolism) and breakdown (catabolism) while pathways related to secondary metabolites correspond with low rates of growth and stress responses<sup>4</sup>. The primary metabolites are generally synthesized continuously during the growth cycle and are necessary for survival and cellular processes like growth and reproduction<sup>6</sup>. They are highly marmaladed across all phyla & kingdoms and are

produced by the bulk of the microbial species with striking structural and functional similarities in sub-micron levels intracellularly due to their swift rates of turnoverability<sup>7,8</sup>. In contrast, secondary metabolites offer survival advantages like improvement in the availability or uptake of nutrients and defense against environmental stress<sup>9,10</sup>. In addition, they display higher levels of accumulation than primary ones either by hoarding inside microbial cells or excreting out into the extracellular media<sup>4,11</sup>. Their synthesis is either mediated by the ribosome (like bacteriocins) or by non-ribosome (like polyketides) with chromosomal genes as their coding regions<sup>12</sup>. Tyc *et al.*<sup>13</sup> examined two main classes of secondary metabolites, depending on their polarity, state of matter and diffusing capacity, as volatile organic compounds and soluble metabolites. The soluble metabolites (polyketides, lipopeptides and bacteriocins) were found to be water-soluble in nature due to their high polarity with more idiosyncratic bio-activities while the volatile organic compounds (indole, pyrazines, and sulphurous compounds) were evaporable molecules, diffusible via pores filled either with air or water.

Species of *Bacillus* are ubiquitous in nature, thriving under diverse environmental provisos and enduring in unusual habitats due to their ability

of sporulation and generation of diverse antimicrobials<sup>14,15</sup> exhibiting immense metabolic and taxonomic diversities along with their ability to produce active metabolites of biological significance like antibiotics, polyketides, cyclic lipopeptides and bacteriocins<sup>16</sup>. This combinatorial approach of traits is superlative in a variety of beneficial attributes which are of paramount significance in the development of pharmaceuticals<sup>17</sup>. Palazzini *et al.*<sup>18</sup> evaluated gene clusters of compounds such as bacillaene, surfactin, amylocyclicin, bacillibactin and iturin (or bacillomycin) in the foundation genomes of strains *B. siamensis*, *B. velezensis* and *B. amyloliquefaciens*. Aleti *et al.*<sup>19</sup> reported the genomic sequences coding for the production of iturin, surfactin, kurstakin and fengycin in the strains of *B. amyloliquefaciens* and *B. velezensis*. The strains of *B. velezensis* were found to host genomic clusters of sequences pertaining to the synthesis of fengycin, bacilysin, difficidin and macrolactin<sup>18</sup>.

A vast sum of literature reviews on different biological activities exhibited by metabolites of *Bacillus* have enlisted antibacterial<sup>20</sup>, antifungal<sup>21-23</sup> and anti-nematodal<sup>24-26</sup> with anti-fungibiosis being the major theme of immense investigations. Metabolites with antifungal activity were also found as potential bio-nematicides with these activities not restricted to spheres of life (such as archaea, bacteria or eukarya)<sup>27</sup>. The cultural filtrates of *B. subtilis* Bbv 57 (KF718836) were found to exhibit bio-control potential with the inhibitory effects on the growth rate of mycelia of *Fusarium oxysporum* and lethal effects on eggs and juveniles of *Meloidogyne incognita*<sup>27</sup>. Similarly, Adam *et al.*<sup>28</sup> examined and confirmed the species of *Bacillus subtilis* as a 'multi-use bacterium' in the control and combat of fungal pathogens and root-knot nematodes as the fungal antagonist and systemic resistant inducer, respectively. With vast accents laid on anti-fungal narrative, less or sparse prominence has been ascribed to the antibacterial efficacy. The present study highlights the efficacy and activity of methanolic extract of *Bacillus vietnamensis*, a marine bacterium isolated from marine sediments, against pathogenic strains.

## Materials and Methods

Sediment sample was collected from the coast of Chidiyatapu (11°29'24.36" N, 92°42'25.24" E), South Andaman in a sterile polyethylene bottle and transported to the laboratory under sterile conditions. 1 g of the sample was aseptically transferred to a

sterile conical flask containing 99 ml filtered sterile seawater and incubated for 3 – 6 h at 37 °C. From this, serial dilutions up to 10<sup>-8</sup> were prepared and 0.1 ml was plated onto the successive Zobell Marine agar plates by spread-plate technique following incubation at 35 °C for 24 h. Post incubation, single, discrete colonies were re-streaked, selected and single colony purity and morphology were observed under the microscope. Pure isolates were maintained as slants, stabs and 10 % glycerol cultures for further analysis.

## Phenotypic and genotypic characterization

The routine biochemical tests were undertaken for the identification of isolates based on phenotypic characters described in Bergey's manual of Systematic Bacteriology<sup>29</sup>. The method of Gram staining was carried out as proposed by Chapin<sup>30</sup> while the biochemical tests were performed along with growth analysis at varying salt concentrations and temperatures. The results were inferred by using Identax Bacterial Identifier (Software version 1.2) with an identification score above 95 %<sup>31</sup>. The genotypic analysis was carried out for 16S rRNA gene amplification by using universal consensus primers 27F and 1492R<sup>32</sup>. The reaction was carried out in the volume of 50 µl and the amplified products were sequenced. The obtained sequences were compared with databases available in databanks and were submitted to NCBI for the designation of an accession number.

## Microbial cultures

Three human bacterial pathogens *Shigella sonnei*, Enteropathogenic *Escherichia coli* serotype (0115) and *Bacillus cereus*, were tested. The isolates were cultured periodically on nutrient agar (NA) and preserved as stabs, slants and 10 % glycerol cultures.

## Screening and identification of bacteria with bactericidal activity

All pure isolates with different morphologies were primarily screened for their bioactivity by cross-streaking method<sup>33</sup> against the three pathogens (*Shigella sonnei*, Enteropathogenic *Escherichia coli* serotype (0115) and *Bacillus cereus*). The test strains were streaked perpendicular across the pathogens in Muller Hinton Agar medium (MHA) and incubated at 37 °C for 24 h.

## Preparation of bacterial crude extract

Potent strains were introduced and inoculated in 250 ml sterile minimal medium supplemented with 2 % sodium chloride (NaCl), 1 % glucose and 1 % yeast

extract followed by incubation at 27 °C for 3 – 5 days under constant shaking<sup>34,35</sup>. Post incubation, the culture was subjected to centrifugation at 11000 rpm for 10 – 15 min followed by ensuing supernatant extorted by equivalent volumes of ethyl acetate and stirred overnight. The resulting extract (MEBV: methanolic extract of *Bacillus vietnamensis*) was then concentrated by using a vacuum evaporator (Buchi, Essen Germany) operated at 40 – 45 °C and the final resulting content was dissolved in the organic solvent (here, methanol; 1 mg/ml as final concentration).

#### Fourier Transform Infrared Spectroscopy (FT-IR)

The functional groups present in the methanolic extract of the bacterial strain were determined by FT-IR spectroscopic measurements. The powdered sample of the extract was diluted with KBr (Potassium bromide spectroscopic grade; mass ratio 1:100) and peak measurements were measured by FT-IR spectrometer (Spectrum Two; Perkin Elmer, USA) at a diffused reflectance mode at 4 cm<sup>-1</sup> resolution.

#### Determination of Total Phenolic Content (TPC)

The TPC of MEBV (25-200 µg/ml) was determined by Folin–Ciocalteu method<sup>36</sup> with slight modifications. Briefly, 0.5 ml MEBV was added to 100 µl Folin–Ciocalteu reagent + 6 ml distilled water and shaken for a minute. Then, 2 ml Na<sub>2</sub>CO<sub>3</sub> (15 %) solution was added to the mixture and was shaken for 30 sec. The resulting solution was then made up to 10 ml by adding sterile distilled water and incubated for 90 min at room temperature. The absorbance was read at 750 nm and the results were denoted as gallic acid equivalents (GE).

#### Reducing power

The reducing power of MEBV was analyzed by following the method of Keshari<sup>37</sup> with slight modifications. The diluted extracts (dilution done in 1.0 ml distilled water) (25 – 200 µg/ml) were mixed with 2.5 ml sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml K<sub>3</sub>Fe(CN)<sub>6</sub> (1 %) and incubated at 50 °C for 20 minutes. Then, 2.5 ml TCA (trichloroacetic acid, 10 %) was added to the mixture and subjected to centrifugation (3000 rpm; 10 min). Following centrifugation, the upper layer (2.5 ml) was extracted and was mixed with 0.5 ml ferric chloride (0.1 %) + 2.5 ml sterile distilled water. The absorbance was read at 700 nm and results were expressed as ascorbic acid equivalents.

#### Free radical scavenging activity

The free radical scavenging capacity of MEBV was evaluated by using DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical<sup>38</sup> with slight modifications. In brief, MEBV (25 – 200 µg/ml) (diluted in ethanol) was mixed in 3 ml DPPH in ethanol (200 µM) and incubated for 30 min under dark conditions. Ascorbic acid was used as a comparative set. The absorbance values were measured at 517 nm and percent inhibition (I %) was computed by using the following equation:

$$I\% = \frac{A_0 - A_e}{A_0} \times 100$$

Where, A<sub>0</sub> = absorbance of blank sample and A<sub>e</sub> = absorbance of the tested sample.

#### Ferric Thiocyanate Method (FTC)

For FTC method<sup>39</sup> under slight variations, the MEBV (200 µg/ml) was mixed with 2.5 % linoleic acid in ethanol (4 ml), 8 ml phosphate buffer (0.05 M, pH 7.0) and 4 ml distilled water and kept at 40 °C under dark. Aliquots (0.1 ml) were mixed with 9.7 ml ethanol (75 %) + 0.1 ml ammonium thiocyanate (30 %). After 3 min, 0.1 ml ferrous chloride (20 mM) in 3.5 % HCl was added to the mixture and the absorbance was read at 500 nm every 24 h until a maximum value was reached. Control and standard (ascorbic acid) setup were run parallel.

#### Antibacterial assay

Antibacterial activity was assessed by the agar well diffusion method as followed by Cherian *et al.*<sup>40</sup>. All pathogenic strains (cell density of 2.0×10<sup>7</sup> CFU/ml) were plated on Mueller Hinton Agar (MHA) and uniform sized wells were punctured onto the agar surface by gel borer. Variable concentrations of MEBV (25, 50, 100, 200 µg/ml) were added to the wells with Gentamycin disc (positive control; 15 mg/ml) and methanol (negative control), separately. Plates were then incubated for 24 h at 37 °C and the diameters of inhibitory zones (mm) were determined.

#### Statistical analysis

All experiments were performed in triplicates and analyzed using one-way ANOVA followed by Duncan's multiple range test (IBM SPSS® 22.0, NY). The *p*-value < 0.05 was considered statistically significant and graphs were constructed by Sigmaplot version 10.1 software (USA).

#### Results and Discussion

The conduits of secondary metabolism in microbial orb is an affluent cause of bioactive secondary

molecules transcribing from aromatics, peptides to isoprenes and polyketides, with their amalgamating trails doling out as one of the few core biochemical metabolics of general metabolism<sup>12,41</sup>. A projected figure of 795 secondary metabolites was found to be synthesized by various species of *Bacillus*<sup>42</sup>, of which antibiotic peptides are one of the most comprehensively revised with their fundamental relevance in medicine and pharmaceuticals.

In the present study, twenty isolates were isolated from the marine sediment and maintained on a nutrient agar medium. The morphologies pertaining to their size, shape and colour were analyzed along with the screening results by cross streaking method depicting one of the isolates, based on various biochemical tests (Table 1), Identax result interpretations and 16S rRNA gene phylogenetic analysis, as *Bacillus vietnamensis* PBChS1 (Accession number MK882498) (Fig. 1).

Table 1 — Table showing results of biochemical tests

Morphology	
Gram staining	+
Motility	+
Colour	Pale yellowish
Biochemical tests	
Catalase	+
Nitrate	-
Citrate	-
Urease	-
Indole	-
H2S	-
Methyl-Red	-
Voges-Proskauer	-
Aesculin Hydrolysis	+
Growth at 0% NaCl	+
Growth at 3% NaCl	+
Growth at 6% NaCl	+
Growth at 8% NaCl	+
Growth at 10% NaCl	+
Growth at 15% NaCl	+
Growth at 4 °C	-
Growth at 20 °C	+
Growth at 35 °C	+
Growth at 40 °C	+
Growth at 50 °C	-
Sugar fermentation:	
Sucrose	+
Dextrose	+
Lactose	-
Fructose	+
Sorbitol	-
Mannitol	+
Inositol	-
Mannose	-
Xylose	-
Arabinose	-
Species identified with % identity	<i>Bacillus vietnamensis</i> , 99 %

A range of varied functional organic groups was reported by the FT-IR spectrum of MEBV (Fig. 2): 3349 cm<sup>-1</sup> (hydroxyl (-OH) group), 2097 cm<sup>-1</sup> (alkynyl C≡C stretch), 1612 cm<sup>-1</sup> (C=O stretch of amide region), 1400 cm<sup>-1</sup> (C-N vibrations of aromatic amines). Also, a moderate stretching was observed in peaks at 1200, 1159, 1113, 961, 886, 660, 603 and 518 cm<sup>-1</sup> related to alkene groups (C=C stretching), polyphenolic compounds, aliphatic amines (C-N stretch vibrations) and alkene groups (C-H stretch), respectively<sup>17,43-45</sup>.

Furthermore, the MEBV demonstrated antibacterial activity with variable inhibition zones against tested pathogens (Figs. 3 & 4). A moderate to high inhibition



Fig. 1 — Neighbour-joining tree of 16S rRNA gene sequences of *Bacillus vietnamensis* PBChS1; *Vibrio furnissii* PBCCW1 (MK973000) was used as out-group

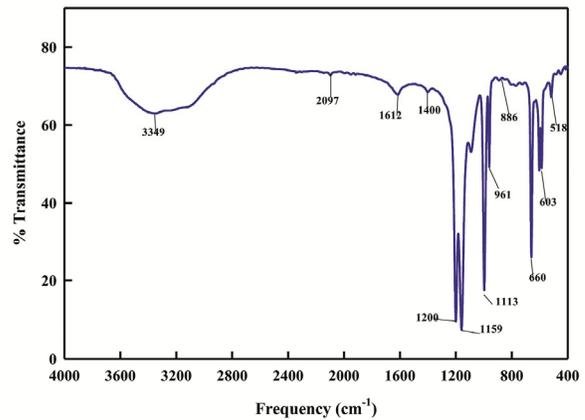


Fig. 2 — FT-IR spectra of MEBV



Fig. 3 — Assessment of antibacterial activity of MEBV by well diffusion assay [(1) 25 µg/ml, (2) 50 µg/ml, (3) 100 µg/ml, (4) 200 µg/ml, and (CL) Control]] against (A) *Shigella sonnei*; (B) *Bacillus cereus*; and (C) *E. Coli*

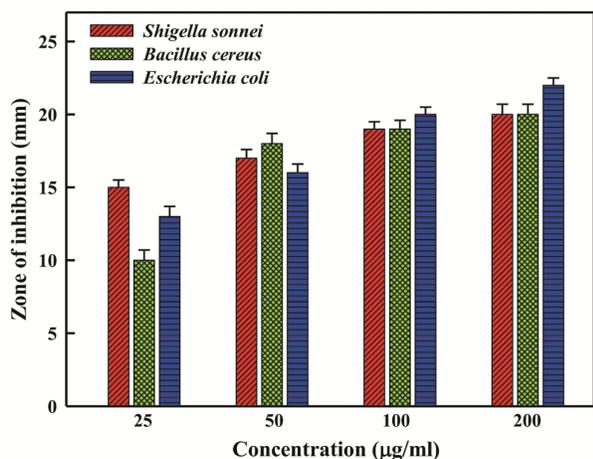


Fig. 4 — Comparative antibacterial activity of MEBV in the concentration range (25 – 200 µg/ml) against pathogenic strains (Results are means ± SD of triplicate measurements,  $p < 0.05$ )

zone was observed against all tested pathogenic strains. The 200 µl extract (50 mg/ml concentration) showed maximum inhibition zone of  $22.3 \pm 0.5$  mm against *E. coli* while an inhibition zone of  $20.0 \pm 0.7$  mm against both *Bacillus cereus* and *Shigella sonnei*. Inhibitory zones were not observed in the case of negative control pure methanol. Cherian *et al.*<sup>40</sup> reported antimicrobial activity of the methanolic extract of *Bacillus aquimaris* against human pathogenic species. A lipopeptide molecule, Tauramamide, isolated and reported from *Brevibacillus laterosporus* PNG276 (Papua New Guinea) was found to be moderately effective against *Enterococcus* sp. and multidrug-resistant *Staphylococcus aureus*<sup>47</sup>. Bacteriocin-type substances released by *Bacillus subtilis* were found to be inhibitory in nature and suppressed the growth of clinical pathogens like *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhi*, *Bacillus cereus*<sup>48</sup> while *Bacillus licheniformis* limited the activities of spoilage bacteria<sup>49</sup>. Ramli *et al.*<sup>50</sup> reported a considerable reduction of biofilm formation in *Burkholderia pseudomallei* due to the presence of N-acyl homoserine lactone in the culture supernatant of *Bacillus* sp. The endospore form of *Bacillus* strain TKS1 restricted and inhibited the growth and occurrence of citrus bacterial canker<sup>51</sup>. The culture broth of *Bacillus mojavensis* B0621A (isolated from *Pinctada martensii*, South China Sea) was found to be anti-fungal (compound Mojavensin A, an iturinic lipopeptide) inhibiting the growth of HL-60<sup>52</sup>. Kalinovskaya *et al.*<sup>53</sup> isolated glyceryl acid derived heptapeptide from marine species of *Paenibacillus profundus* SI79 found to be inhibitory against pathogenic species of *S. epidermis*, *S. aureus*,

Table 2 — Table showing total phenolic content in MEBV

S No.	MEBV concentration (µg/ml)	Total phenolic content (GE mg/ml)
1.	25	$0.72 \pm 0.23$
2.	50	$1.24 \pm 0.16$
3.	100	$1.47 \pm 0.31$
4.	200	$1.68 \pm 0.22$

*Enterococcus faecium* and *B. subtilis* and cytotoxic to SK-MEL-28 cell line. The cyclic peptide molecules of YM-266183 and YM-266184 isolated from *Bacillus cereus* were found to be effective against pathogenic staphylococci and enterococci<sup>54,55</sup>.

Large assemblies of secondary metabolites belonging to polyketide and lipopeptide miscellany are produced by the species of *Bacillus subtilis* and *Bacillus amyloliquefaciens* slicing out the substantial fraction of their observed metabolite diversities<sup>19</sup>. About 4 % of the total genome of *B. subtilis* is allotted to the synthesis of bacteriocins, polyketides, Non-Ribosomal Peptides (NRPs) and antibiotics<sup>46</sup>. Further, the complete absence or minimal order of lipopeptides and polyketide production in species populating other spaces of the environment is a pinpoint of the role amused by these secondary metabolites in assorted associative habitations<sup>19</sup>. Various formulations of cell-free or whole-cell supernatants of *Bacillus* species have been alluded as potential biopesticide<sup>56,57</sup>. Mendoza *et al.*<sup>25</sup> assessed the anti-nematobiosis activity of cell-free filtrates of *B. firmus* against important agricultural nematodes (of stem, burrowing and root-knot types) resulting in the significant nematodal paralysis followed by increased rates of mortality and reduced egg hatching. A similar report was stated by Xiong *et al.*<sup>58</sup> in reduced egg hatching of *Meloidogyne incognita*. The metabolic nucleic bases of uracil, 9H-purine and dihydrouracil synthesized by *B. subtilis* and *Bacillus cereus* were found to be nematocidal in nature activity against *Meloidogyne exigua*<sup>59</sup>. Yanfei *et al.*<sup>60</sup> evaluated the bio-nematocidal efficacy and activity of *Bacillus subtilis* against nematodal species of *Meloidogyne javanica* inferring the presence of 5'-phosphoribosyl-N-formyl-glycinamide synthase II (FGAM) encoded by purL gene, as the reason of its nematocidal activity.

It is generally considered that the compounds of phenols and their derived origins possess promising anti-oxidative activities<sup>61</sup>. The phenolic content in MEBV was found to be in the range of  $0.72 \pm 0.23$  –  $1.68 \pm 0.22$  GE mg/ml (Table 2); the former increased with increased concentrations of MEBV. The reductive ability of MEBV in the  $Fe^{3+}$ -  $Fe^{2+}$

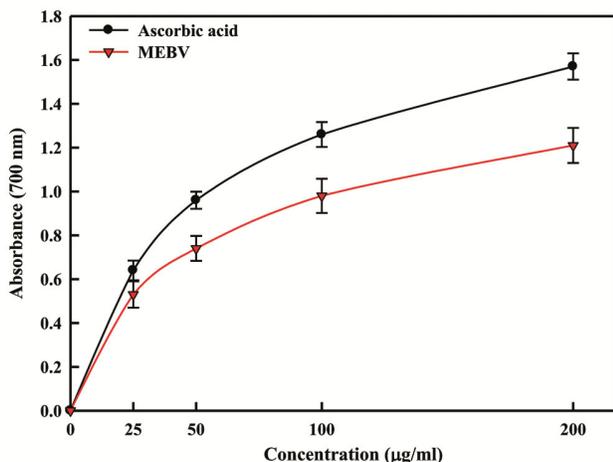


Fig. 5 — Reducing power of MEBV (Results are means  $\pm$  SD of triplicate values)

transformation was investigated and compared with ascorbic acid. The reducing power of MEBV amplified with increasing concentrations exhibiting high activity (Fig. 5). The reducing capacity of a compound may serve as a major indicator of its anti-oxidative potential; generally coupled with the presence of reductones<sup>62</sup>. The mechanistic action of reductones involves the breakage of the free radical chain via splitting of a hydrogen atom or by the prevention of peroxide formation<sup>63</sup>. It is deduced that phenols may act well by the donation of electrons and react with free radicals; converting into more stable products leading to the termination of free radical chain reaction comparable as that of reductones.

The free radical chain reaction is regarded as the general mechanism of lipid peroxidation. The scavenging effect of MEBV indicated a conspicuous effect on scavenging free radical potential (Fig. 6) with increasing concentrations. The concentration of 200  $\mu\text{g/ml}$  exhibited the strongest scavenging activity among the tested concentrations. A radical scavenger usually reacts and quenches peroxide radicals leading to the termination of the peroxidation chain reaction. The ion of DPPH is a stable free radical accepting an electron and forming a stable diamagnetic molecule<sup>64</sup>. The decrease in absorbance infers the reductive capability of DPPH induced by antioxidants.

The levels of peroxide during the initial stage of lipid oxidation were measured by the FTC method. During the oxidation of linoleic acid, the peroxides react with  $\text{Fe}^{2+}$  to form  $\text{Fe}^{3+}$  with the latter forming an  $\text{SCN}^-$  complex readable at a maximum absorbance of 500 nm<sup>65</sup>. The lipid peroxidation inhibition effect of

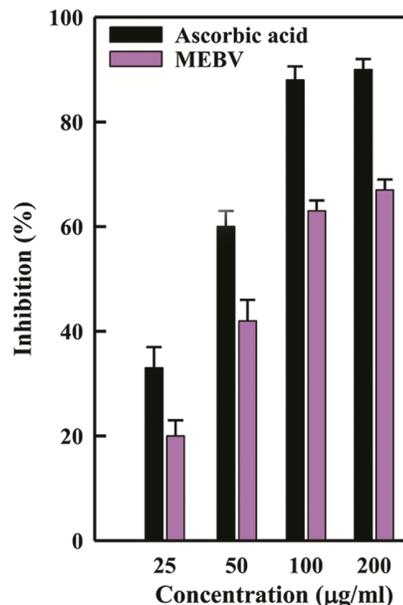


Fig. 6 — Free radical scavenging activity of MEBV analyzed by DPPH method (Results are means  $\pm$  SD of three parallel measurements,  $p < 0.05$ )

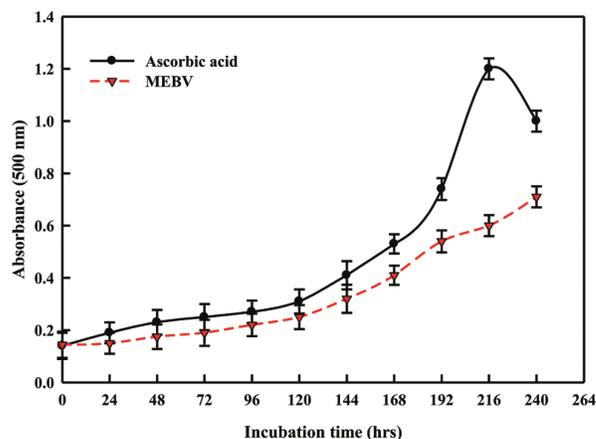


Fig. 7 — Antioxidant activity of MEBV as quantified by FTC method (Results are means  $\pm$  SD of triplicate values)

MEBV is presented in Figure 7. After the incubation period of 216 h, an impediment in the peroxide formation was observed due to the non-availability of linoleic acid<sup>65,66</sup>. The intermediate products were converted to stable end products leading to the cessation of  $\text{Fe}^{2+}$  oxidation and decreased absorbance at 500 nm. Overall, the results exhibited an effective anti-oxidative potential owing to its phenolic content and reducing power.

Hence, the marine-derived *Bacillus* amounts to a promising 'hotspot' of distinctive molecules with sizeable therapeutics prospects. Regular and routine

biological assays typically lay emphasis on cytotoxic and antimicrobial activities whereas more effectual and safer drug designing warrants conjugational advances with many pharmacological active biological compounds. Thus, it is suggestive to expand and widen the domain of biological screening and presumptive identification for the detection and determination of incomparable and seldom probed bio-activities important for the remedial therapy of chronic medical ailments.

### Conclusion

The present study typifies the antibacterial activity of marine bacteria *Bacillus vietnamensis* (its methanolic extract) against pathogenic strains warranting more structured and sophisticated insights for its practical procedures of application in drug pharmacology and their amendments. The comparative and coupled briefings on their synthetic pathways, nature of compounds and their activity and efficacy complied with the realizations of genetic affirmations are reasonably necessitated for safe exercise in realistic applications. Further, the studies on assumed synergistic effects of these bio-active mixtures alone or within themselves or in conjugation with other chemical species need to be addressed in a convenient way to summarize the overall settings in medico-dynamics and related therapeutics.

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### Conflict of Interest

The authors declare no conflict of interest.

### Author Contributions

Conceptualization and methodology: TC & RM; data curation: TC, SE & RM; formal analysis: TC, WJ & RM; investigation: TC, SY & RM; resources: RM; supervision: RM; validation: TC; and roles/writing – original draft and writing – review & editing: TC, WJ, SE, SY & RM.

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