ABSTRACT

Mangroves are growing in extreme conditions of the marine environment and the microorganisms present in the environment have to be highly tolerant of the extreme conditions. Therefore, we expected that the microorganisms from mangrove soil may be the potential source of bioactive compounds. We aimed to investigate the Bacillus flexus from mangrove soil, analyze the morphology, cultural and genotypic identification by using 16S rRNA technique. In addition to identify the novel compound by (GC-MS) Gas Chromatography Mass Spectrum from mangrove soil. The identified potential bioactive compounds were docked against breast cancer responsible pathway protein.

KEYWORDS: Bacillus flexus, 16S rRNA, GC-MS, Phylogenetic tree, Breast cancer, Mangrove soil.

INTRODUCTION

The genus Bacillus is widely distributed in nature and most commonly found in soil. Bacillus is a large and very diverse group of organisms. It comprises of gram-positive, endospore forming, aerobic bacteria, rod-shaped and most closely related to the genera Listeria.[1, 2] Kingdom Bacteria; Phylum Firmicutes; Class Bacilli; Order Bacillales; Family Bacillaceae; Genus Bacillus; Species Bacillus flexus,[3] Bacillus species have been some of the first bacteria ever characterized; their relationships to one another remain enigmatic.[4] Bacillus includes both free-living and pathogenic species, it is a ubiquitous in nature, it has been isolated from environments as diverse as fresh water, saline water, soil, plants, animals, and air.[5] The Bacillus has been harnessed by industry for the production of molecules such as
riboflavin, Streptavidin, lactamase.\[6\] *Bacillus* species preeminent hosts heterologous protein production.\[7\] It can be considered as a dark horse in the race to generate sustainable energy, ecofriendly non-fossil fuel-based polymers and bioactive molecules for therapeutic use.\[8\]

The isolation and characterization *Bacillus* species sequence of 16S rRNA gene was used for this purpose as it has been widely employed to estimate the relationship between the phylogeny and also it is most commonly used as a rapid tool for the identification of unknown bacteria up to the species level.\[9\] The 16S rRNA gene was amplified by PCR using the DNA of the organism to be identified as template.\[10\] which provides a measure of genomic similarity above the level of species allowing comparisons of relatedness across the genus.

Cancer was a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread was not controlled, it can result in death. The cancer was caused by both external factor and internal factors. Cancer was the general name for a group of more than 100 diseases. Cancer was a complex genetic disease and major leading cause of death.

Breast cancer is the second leading cause of cancer death in women only next to lung cancer considering the lacunae of reliable and potential drugs.\[11\] Common cause of cancer incidence (115,251 cases) and cancer deaths (53,592 deaths) in women in India.\[12\] It was a type of cancer where cells in the breast divide and grow without normal control. The incidence of breast cancer has doubled during the past 30 years.\[13\] In India, around 555,000 people died of cancer in 2010, according to estimates published in The Lancet today (March 28, 2012), breast cancer can have a substantial impact in India in averting future cancer deaths.\[14\] In India annually 22\% of cancer cases and 17\% of the cancer deaths in women were occurring. More than 50\%-80\% of breast cancer patients in different regions of India present in advanced clinical stages.\[15\]

NF-κB (Nuclear Factor-KappaB) is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors.\[16\] The Rel/NF-κB family of transcription factors is involved mainly in stress-induced, immune and inflammatory responses. These molecules play an important role during the development of certain homeopathic cells, keratinocytes and lymphoid organ structures. More recently, NF-κB family members have been implicated in neoplastic progression and the formation of
neuronal synapses. NF-kB is also an important regulator in cell fate decisions, such as programmed cell death and proliferation control, and is critical in tumorigenesis.\textsuperscript{[17]}

Molecular docking or \textit{Insilico} docking is a method which predicts the preferred conformations of one molecule to a second one when bound to each other to form a stable complex. Molecular docking can be considered as a dynamic procedure where the correct geometry of a “key” is sought which will open the “lock”.\textsuperscript{[18, 19, 20]}

The marine microbes remain one of the most potent sources of pharmaceutically important compounds and have recently emerged as a new source of structurally novel natural products for the development of new drug candidates.\textsuperscript{[21]}

\textbf{MATERIALS AND METHODS}

\textbf{Sample Collection}

The samples collected from Velar estuary region (Lat. 11°25’N and Long. 79°49’E), mangrove soil sediment. Bacterial cultures were isolated from the soil. Isolations were carried out after serial dilution of samples (1 gm) in saline (9ml). One hundred microliters of serially diluted samples were spread plated on Zobell’s Marine Agar (ZMA) plates (Himedia, India) and plates were incubated at 30°C for 24-48 h. The colonies developed were isolated, purified by streaking and pure colony was transferred on to agar slant maintained in ZMA.

\textbf{DNA Extraction}

The Estimation of biomass cells was harvested by centrifugation at 7000 RPM for 15 min, washed with distilled water and dried at 80°C in airflow dried to a constant weight. 1.5ml of overnight culture was taken in an Eppendorf tube. It was centrifuged at 12,000 rpm for 2 minutes. Supernatant was discarded and to the pellet 500μl of Solution I was added. It was incubated at 37°C for 3 hours. Equal volumes of Phenol: Chloroform – 24:1 was added to the supernatant. The Eppendorf tube was centrifuged at 12,000 rpm for 15 minutes at 4°C aqueous phase was transferred to a fresh Eppendorf tube. 1/10\textsuperscript{th} volume of 3M sodium acetate was added. Twice the volume of 99.9% Ethanol was added to the aqueous phase. Invert mixed slowly. Overnight incubation was performed in -20°C (to enhance DNA precipitation). It was centrifuged at 12,000 rpm for 15 minutes at 4°C. The pellet was washed with 70% ethanol. It was centrifuged at 12,000 for 10 minutes at 4°C. Supernatant was discarded and the pellet was air dried. The pellet was suspended in 20 μl of 1X Tris EDTA buffer. Quantitative Determination of DNA done by Spectrophotometric Method.
Agarose gel electrophoresis 0.8% Agarose, 50X TAE buffer (Tris base-242g/l, Glacial acetic acid-57.1ml/l, 0.5M EDTA-100ml/l for 50X), 6X gel loading dye (Xylene cyanol blue-0.25% and Glycerol30%) and Ethidium bromide solution (0.5mg/ml). The PCR reaction mixture contained [25μl of Master Mix contains: 10X Taq buffer, 2mM Mgcl2, 0.4mm dNTP mix and 2U Proof read Taq DNA polymerase]. Forward and reverse primer “GCTGCGTTCTTCATCGATGC” and “GGAAGTAAAAGTCGTAACAAGG" reaction was carried out by an initial denaturation of 95°C for 3 min followed by 35 cycles of denaturation at 94°C to 4 Sec, annealing temperature of 46°C for 45 Sec and elongation at 72°C for 15 min. After the completion of reaction amplification was confirmed by gel electrophoresis.

**Phylogenetic analysis**

The 16S rRNA region of the marine *Bacillus* strains was sequenced and the sequential result obtained was matched with existing sequences available in NCBI (National Centre for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool).[^22] Phylogenetic analyses were carried out by using Molecular Evolutionary Genetics Analysis MEGA 5.0 software.[^23] Sequences were aligned by Clustal W Multiple Sequence Alignment Program and analyses. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model.[^24] The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed.[^25] Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed.

**GC-MS Analysis**

The GC-MS analysis of the crude compound of *Bacillus flexus* was recorded in GC clears 500 Perkin Elmer instrument. The crude compound obtained was GCMS analysis in order to find out its chemical constituents. GC conditions used were as follows: column Elite-1 (dimethyl poly siloxane 30 m lengths, 1 μm thickness and 0.25 mm in diameter. Injector temperature was 250°C carrier gas used was helium at 1 ml/min; Initial temperature of the oven was 110°C, increased from 110°C up to 200°C at the rate of 10°C per minutes, 200-280°C at the rate of 5°C per minute and final hold time was at 280°C for 9 min.

**Protein Data Bank (PDB)**

The Protein Data Bank (PDB) is a repository for the 3-D structural data of large biological molecules, such as proteins and nucleic acids. The 3-D crystal structure of the targeted breast
cancer protein BRCA1 [Nuclear Factor Kappa-B (NF-kB) (ID: 1NFK)] was retrieved from the protein data bank (PDB) (www.rcsb.org/pdb). Structural and active site studies of the protein were done by using the tools CASTp server (Computed Atlas of Surface Topography of Proteins).

GLIDE
GLIDE is one of the modules in Schrodinger package which is usually used for the docking studies. This glide has searched for the favored region for protein-ligand docking with Hydrogen bond interactions. Glide can be run in rigid or flexible modes. In the docking study flexible docking was performed, the ligand tried to bind with different conformers. For each core conformation has search possible locations and an orientation is performed over the active site of the protein. GLIDE XP (Extra-precision) model is a refinement tool. This XP mode is designed for using best ligand poses. XP mode has only the top-scoring ligands docked.

RESULT
The bacteria were obtained from the mangrove soil, from velar estuary. The isolate was initially identified as Bacillus species because it was gram-positive, catalase positive, motile, endospore forming, rod shaped and aerobic bacterium (Fig: 1), the genomic DNA was used for the amplification of 16S rRNA gene by PCR and a comparative search for this sequence revealed 99% homology to Bacillus flexus (Gen Bank Accession Number: KC 964543). Further, multiple sequence alignment was done using clustal W programme. Based on this, phylogenetic tree was constructed using the MEGA 5 software (Fig: 4). The position of the isolated strain within the same group of species based on 16S rRNA gene was analyzed by the tree constructed using maximum likelihood method the sequence similarity with the species was 99% other closely related species of Bacillus flexus.

The ligand and target protein was geometrically optimized and docked using Schrodinger software. XP mode is designed for using best ligand poses. XP mode has only the top-scoring ligands should be docked. The docking simulations in the active sites of 1NFK were performed, which have been shown to successfully reproduce experimentally observed binding modes in terms of lowest docking energy. The target protein structure of Nuclear Factor Kappa-B (1NFK) was docked with Bacillus flexus derived compounds which provided excellent results as were seen by the least value of the binding energy. The best possible
binding interaction modes of the compounds at targeted protein’s active sites are displayed in the Fig: 10.

The docked ligand molecules were selected based on docking energy and good interaction with the active site residues and the results are displayed below.

Fig: 1. A- *Bacillus* grown on agar plate B- Agar slant C- Gram staining 100X

Genomic DNA amplification of 16S rRNA gene by PCR

Fig: 2. Agarose gel electrophoresis of 16SrRNA band

T- Tube 1 & Tube 2: 810bp of bacterial 16S rRNA sequence

M- Marker: 250bp DNA ladder: 100bp, 250bp, 500bp, 750bp and 1000bp
Submitted sequence of *Bacillus flexus* in Gen Bank

Accession: KC964543

Source: *Bacillus flexus*

gagcgacagatggagagttggctccctgtatgtagcgcggcgcggcgtgagtaacgtgtaacctgcc
ttaagactgggataactccggggaacccggctaataccggatggtgtgtggaaccgcaggtccttaaac
taccaaggcaacgatgcgtacgccacctgagaggtgatcgccacactgggactgagaacagggcccag
actctacggggaggcagcagtgaatctttccgcaatgacagaaagtctgacgcgagcagcgacgcggtgag	
tgatgaaggttttcggatctgtaaagctcttgtgtaggggaagaacaagtaacctctgtcagatagggcggta
cctgaagagttaactcaaccgaaaccacggctaatacgtgcacagccacggtaataatcgtaggtgcca
agcgtgtgcggaattttggcctttaaggcgtgggtggttttcttaattgcttgatgtgaaagcccccg
gctcaaccggggagggtctggaactttggtggtgtagtgagaacgggactgatccacgacgtatcgtaggc
tacggggaattggagagttggcagcagaaaacactgacagcgcgacgtcgtgatggtgacgctgctggatcctttggttccccccttattaggtggtcgc

Fig: 3. 16S rRNA Sequence of *Bacillus flexus*
Sequence information

Table: 1. Sequence information of *Bacillus flexus*

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<th>Properties</th>
<th>Description</th>
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<td>Length</td>
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<tr>
<td>Organism</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Name</td>
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</tr>
<tr>
<td>Description</td>
<td><em>Bacillus flexus</em> strain psrm 16S ribosomal RNA gene, partial sequence</td>
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<table>
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<tr>
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<tbody>
<tr>
<td>Adenine (A)</td>
<td>203</td>
</tr>
<tr>
<td>Cytosine (C)</td>
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</tr>
<tr>
<td>Guanine (G)</td>
<td>263</td>
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<tr>
<td>Thymine (T)</td>
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<tr>
<td>C + G</td>
<td>446</td>
</tr>
<tr>
<td>A + T</td>
<td>364</td>
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<p>| | |</p>
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<th></th>
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<tr>
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<td>0.226</td>
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<tr>
<td>0.325</td>
<td>0.199</td>
</tr>
<tr>
<td>0.551</td>
<td>0.449</td>
</tr>
</tbody>
</table>

Phylogenetic tree

![Phylogenetic tree](image)

**Fig: 4. Phylogenetic evolution of *Bacillus flexus***

Gas chromatography Mass Spectrum (GC-MS)

The structural elucidation of the compound was based on the results of GC-MS analysis, retention time (RT) peaks (9.33, 9.97). The spectrum was comparable to the standard report data. The retention time peak 9.33, 9.97 was referred to the compound 1, 2-Benzenedicarboxylic acid, butyl Octyl ester and 1-Oxacyclopentadecan-2-one, 15-ethenyl-15-methyl. Based on these spectral data the possible structure of the active principle was elucidated with the help of chemsketch. The Properties and 2D structure of the compound obtained from the GC-MS analysis was given in the Table: 2. and Fig: 8.
GC-Mass Spectrum analysis of *Bacillus flexus*

Fig: 5. Retention time of the *Bacillus flexus*

Fig: 6. Retention time peak value 9.33 denotes 1, 2-benzenedicarboxylic acid, butyl Octyl ester

Fig: 7. Retention time peak value 9.97 denotes 1-oxacyclopentadecan-2-one, 15-ethenyl-15-methyl
Linear structure of the GC-MS compound

![Linear structure of the GC-MS compound](image)

Fig: 8. 2D representation of ligand molecules

Properties of chemical compound

Table: 2. Description of chemical properties of the compound

<table>
<thead>
<tr>
<th>Properties</th>
<th>1, 2-benzenedicarboxylic acid, butyl Octyl ester</th>
<th>1-oxacyclopentadecan-2-one, 15-ethenyl-15-methyl</th>
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</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C_{20}H_{30}O_{4}</td>
<td>C_{17}H_{30}O_{2}</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>334.4498 [g/Mol]</td>
<td>266.418915 [g/Mol]</td>
</tr>
<tr>
<td>H bond acceptors</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>H donors</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Protein Structure

![Protein Structure](image)

Fig: 9. 3D Structure of the target Breast Cancer Nuclear Factor Kappa-B (NF-kB) Protein

Protein and ligand docking

The Bioactive compound were derived from *Bacillus flexus*. The protein responsible for breast cancer Nuclear Factor Kappa-B (1NFK) was docked with the GC-MS compound. The docked ligand molecules were selected based on docking energy and good interaction with
the active site residues. The ligand poses energy -16.1462 kcal/mol for 1, 2-benzenedicarboxylic acid, butyl octyl ester and -10.9537 kcal/mol for the 1-oxacyclpentadecan-2-one, 15-ethenyl-15-methyl. And also the protein was docked with available drug; the docking energy result values were compared.

Fig: 10. Interaction of protein and ligand molecules

Fig: 11. 2D representation of the 1, 2-Benzenedicarboxylic acid, butyl Octyl ester and receptor interactions
Docking interaction with available drug (Pazopanib)

![Docking Interaction Image]

**Fig: 12. 2D and 3D docking interaction of available drug (Pazopanib) with target protein 1NFK**

**Comparison of docking energy**

**Table: 3. Comparison of docking energy value**

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Docking Energy Level (Kcal/Mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2-Benzenedicarboxylic acid, butyl Octyl ester</td>
<td>-16.1462 kcal/Mol</td>
</tr>
<tr>
<td>1-oxacyclopentadecan-2-one, 15-ethenyl-15-methyl</td>
<td>-10.9537 kcal/Mol</td>
</tr>
<tr>
<td>Pazopanib (Available drug)</td>
<td>-10.9352 Kcal/Mol</td>
</tr>
</tbody>
</table>

**DISCUSSION**

*Bacillus* are “Generally Recognized as Safe” (GRAS) organism.[26] It is not a cause of concern because its release into the environment would not be a hindrance. *Bacillus* is a well-known, industrially robust organism. It can be easily exploited for a wide range of novel biotechnological applications, production of biofuels, biopolymers, and antimicrobial agents. Basically mangrove derived compounds are ecofriendly and safer for medicinal value application. Natural products and their derivatives have been invaluable as a source of therapeutic agents it possess high chemical diversity, biochemical specificity and molecular diversity within the boundaries of reasonable drug-like properties.[27, 28]

The study isolation and molecular identification of marine soil bacteria using the samples were spread plated on Zobell’s Marine Agar (ZMA). The culture plate, agar slant and gram staining image of *B. flexus* was shown in Fig: 1. The screening of *B. flexus* has gained more commercial importance of the organism.
The genomic DNA isolation reagent (Genomic DNA purification kit, PCR Master Mix, Agarose gel electrophoresis consumables and Primers) the electrophoretic separation were carried out in 0.8% agarose, 6X loading dye and ethidium bromide solution. Separation of the agarose gel is movement caused by the gel matrix. The strain was amplified through PCR the molecular weight 0.810kb corresponding the genomic DNA ladder 0.8% agrose gel image was shown in Fig: 2. The amplified 16S rRNA gene nucleotide sequence length was 810bp (Fig: 3). The sequence was performed in BLAST and the sequence revealed 99% homology to *Bacillus flexus*. The sequence was submitted in (Gen Bank Accession Number: KC 964543). The multiple sequence alignment was performed, the use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy, the phylogenetic tree was constructed with 16S rRNA sequencing was shown in Fig: 4.

Structural elucidations of the compound were done by Gas Chromatography Mas Spectrum (GC-MS) analysis Fig: 5 [30]. GC/MS is a method for identification of more compounds and detection from different Bacillus *sp.*[31] the Retention Time (RT) peaks (9.33, 9.97) were shown in Fig: 6, 7. The spectrum was comparable to the standard report data. The retention time peak 9.33, 9.97 was referred to the compound (Staflex BOP) 1, 2-Benzenedicarboxylic acid, butyl Octyl ester and 1-Oxacyclopentadecan-2-one, 15-ethenyl-15-methyl (Fig: 8). Here we performed an *Insilico* docking method. Derived 3D ligand molecules were docked with homology model protein Nuclear Factor kappa-B (NF-kB) (ID: 1NFK) (Fig: 9) responsible for breast cancer. The docked ligand molecules were selected based on docking energy and good interaction with the residues and the results are shown in (Fig: 10, 11) and table: 3. The NF-kB protein was also used in the comparative docking analysis with commercial drug Pazopanib,[32] and the result is shown in Fig: 12. The docking score value is -16.1462 kcal/Mol for (Staflex BOP) 1, 2-benzenedicarboxylic acid, butyl Octyl ester and the results show the least docking score value was observed than available drug pazopanib. Drug discovery from natural resource has played an important role in the treatment of cancer and indeed, most new clinical applications towards combating cancer therapy.[33]

**CONCLUSION**

The present work reveals the identify a novel potential bioactive compound inhibiting the breast cancer from the mangrove, soil sample at Velar Estuary, Parangipettai, Tamilnadu, India. The genotypic identification was carried out by 16S rRNA technique. The 16S rRNA
sequence, comparative searches confirmed that the BLAST result, 99% homology to *Bacillus flexus*. This sequence was submitted to NCBI Gen Bank accession number KC964543.

*Bacillus* species have defended their position as dominant bacteria in industrial fermentations as advances have been made in recombinant DNA technology. New industrial enzymes produced by *Bacillus* strains have emerged as a result of mutations, cloning and protein engineering.

The GC-MS analysis revealed two Bioactive compounds were (Staflex BOP) 1, 2-Benzenedicarboxylic acid, butyl Octyl ester and 1-Oxacycloptadecan-2-one, 15-ethenyl-15-methyl. The chemical structural characteristics revealed that these molecules are having amphiphilic properties as well as flexible and rigid pharmacophoric segments. Their presence of a flexible segment paves a challenging field for conformational analysis exploring of putative bioactive conformations. The 3D structure for homology model human NF-kB was predicted. The structure of bioactive compound would be used for structure based drug discovery.

Two lead molecules were identified through ligand based virtual screening and computational docking. These leads had good binding affinity towards NF-kB. All potential inhibitors had good ADMET (Absorption, Disruption, Metabolism, Excretion and Toxicity) properties. Commercial drug Lead 1 belongs to pazopanib (Commercial) is an established kinase inhibitor used for cancer treatment, Lead 2 Staflex BOP and Lead 3 (1-Oxacycloptadecan-2-one, 15-ethenyl-15-methyl). Analysis of binding orientation of Lead 1, Lead 2 and Lead 3 with NK-kB. The docking complex had good interaction through hydrogen bonds, van der Waal contacts with active site residues. Hence, Lead 1 molecule was commercial drug docking score -10.9352 kcal/Mol. Lead 2 and Lead 3 with docking score -16.1462 kcal/mol and -10.9537 1462 kcal/Mol. From the comparison of docking score value the Lead 2 has lesser than Lead 1 molecule. So it would be useful for designing an inhibitory drug molecule for cancer treatment.

Further research is needed for refinement to enrich the activity of the ligands and inhibiting mechanism of the breast cancer protein, especially in the animal model system and also to determine the dosage of safety levels, in order to explore this promising avenue for breast cancer control and ensure the healthy state of women.
ACKNOWLEDGEMENT
We are thankful to the authorities of UGC-SAP department of Zoology, Annamalai University for providing necessary facilities to carry out this work.

REFERENCE


