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Research Article

ISOLATION AND CHARACTERIZATION OF ANTICANCEROUS BIOACTIVE COMPOUNDS FROM MARINE *BACILLUS SUBTILIS*

N. Praveena Kumari[1], M. Raghu Ram*[2]

[1]*Department of Microbiology, Govt College for women (A), Guntur, Andhra Pradesh*

[2]*Department of Microbiology, Acharya Nagarjuna University, Guntur, Andhra Pradesh*

**Corresponding author: mraghuram2002@gmail.com*

ABSTRACT

In the present research, soil samples were collected from Nijampatanam marine sediments and isolated bacteria were identified through 16s rRNA and mass production of bioactive metabolite was carried out by optimizing physic-chemical parameters such as temperature, incubation, NaCl concentration, carbon and nitrogen sources. Chromatographically purified crude compound was structurally identified and biological analysis like anticancer properties in both *in vitro* and *In silico* were done with MTT assay and auto dock 4.2 against human, breast cancer cell line. Out of 10 distinct isolates, isolated from Nijampatanam marine sediments, only one; MRRPK4, exhibited considerable amount of anticancer activity. Molecular sequence shows that the isolated bacterium belongs to the genus *Bacillus* and species *subtilis sub sp inaquosorum* with the genbank accession number MT421945. Mass production of biologically active compound achieved by using 60hr incubation, 35°C temperature, pH 7 and NaCl concentration 5.0 %. 1.0 % fructose and peptone have shown excellent growth and production. Ethyl acetate serves as a suitable solvent for extraction and chromatographically purified compound shows a novel structure (3-(3- methylbutan-2-yl) octahydropyrrolo(1,2-a)pyrazine-1,4,dione) with the molecular weight of 238g/mol. Purified compound showing concentration dependent antioxidant activity. *In vitro* MTT assay of purified compound shows concentration dependent activity against MCF-7 cell lines with IC_{50} values of 12.32µg/ml. This activity was confirmed by docking of purified compound with proteins of above tested cancer cell lines (HER2 proteins) and standard drug doxorubicin yields similar results with greater binding energy, inhibition constant and greater number of amino acid interaction with hydrogen bonds.

Keywords: Antioxidant, MTT-Assay, Docking, Doxorubicin, Bioactive.

1. INTRODUCTION

Nature remains an important pool for microorganisms that are capable of producing novel antimicrobials [1]. Marine bacteria represent an unexplored pool for the discovery of unique natural products with diverse activities [2]. Production of antimicrobial compounds by marine bacteria has been well known for long time. *Bacillus* species are useful in many pharmaceutical, medical, and industrial processes as they are a host of antibiotics, and other biologically active metabolites [3]. *Bacillus species* like *B. subtilis*, *B. brevis*, *B. polymyxa*, *B. circulans*, *B. licheniformis*, *B. cereus* are known to produce potential antibiotics exhibiting a wide range of activities like anti-viral [4], anti-fungal [5], anti-mycoplasma [6] and anti-ameobocytic [7] that are being recognized over five decades [8]. Studies have shown that the genus *Bacillus* produce above 45 antimicrobial molecules having clinical value while some are assayed *in vitro* to control food microbes and plant diseases [9, 10] and few of them are known to produce fatty acids that have different biological properties. These compounds mediate chemical defense against microorganisms [11] and also protect against oxidative stress by exerting an antioxidant role [12].

These bioactive compounds possess bactericidal and bacteriostatic properties against a wide range of bacteria [13]. Long-chain eicosapentaenoic acid and docosa-hexaenoic acids inhibit the proliferation of breast cancer cell lines in animal experiments [14]. Cancer is the second leading cause of deaths in men and women worldwide. The reduction in mortality and morbidity is being achieved among cancer patients through the use of current chemotherapeutic agents. However, the application of many anticancer drugs is associated with

high toxicity due to their mechanisms of action and non-specific targeting. Current anti-estrogen medicine tamoxifen is widely used in the prevention and treatment of estrogen receptor positive breast cancer [15] causes severe side effects. A molecular docking study conducted on phyto-estrogens from *Asparagus racemosus* determined a multi-targeted nature of phytoestrogens for inhibiting breast cancer progression [16, 17]. Molecular docking analyses were also employed to find the possible targets of steroidal saponins of *Dregea sinensis* Hemsl for its anti-cancer activity [18]. Interactions of different saponins of Vietnamese ginseng (*Panax vietnamensis*) with tumor necrosis factor-α (TNFα) were also studied using *in silico* approaches [19]. However, there are no reports available portraying the binding ability of pyrazine derivatives to target proteins involved in cancer pathways. Identification of potential protein targets involved in the process of malignancy and the binding ability of bacterial derived pyrazine to the selected targets would ravel the path to the development of anti- cancerous drugs.

In the present study, characterization of bioactive compounds from marine bacteria isolated from Nijampatanam marine sediments Andhra Pradesh India, and its potential *in vitro* and *in Silico* approach was adopted to identify the targets and to expose the mechanistic binding pattern of pyrazine in order to explore its potential as an anti-cancer properties. Considering various parameters obtained from docking studies, it is possible to derive insights on the binding interactions. Finally, the properties of pyrazine derivatives for its use as a drug and aftermath of its intake.

2. MATERIAL AND METHODS

2.1. Cell lines, Chemicals and reagents

The chemicals, culture media and antibiotics used in the present research were purchased from HiMedia Laboratories. Thin layer chromatography (TLC, Silicagel 60) plates from Merck Ltd. Pathogenic strains were obtained from microbial type culture collection centre (MTCC). MCF-7 cell lines were procured from the National Center for cellular Sciences, Pune.

2.2. Isolation and molecular characterization of Marine Bacteria

Soil samples were collected from Nijampatanam, Rapalle, India. The bacterial strains were isolated on nutrient agar medium [20]. Antibacterial screening was carried out to select the potential isolate. Molecular identification was carried out by 16S rRNA partial gene sequencing [21]. Sequencing was carried out at NCIM Pune and analyzed with Gen Bank database (http://www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (BLAST) tool. Phylogenetic tree was constructed with neighborhood joining method using Mega5.0 software [22].

2.3. Screening of antibacterial activity

Isolates were tested for their antibacterial activity against *Escherichia coli*. The activity was carried out by agar well diffusion method. Overnight culture broth of bacterial isolates from the marine soil was loaded in to well of pre-poured Muller and Hinton agar plates seeded with indicator test bacteria. Plates were incubated at 37°C for 24h and observed for clear zone around the well. Antibacterial activity was expressed in terms of clear zone produced around the well after 24 hr incubation.

2.4. Optimization of production medium

About 100 ml of sterile nutrient broth were inoculated with 1% of 24hr old MRRPK4 culturein 3 replicates and incubated at different incubation periods (24, 36, 48, 60 and 72 hrs), temperatures (20°C , 25°C , 30°C , 35°C , 40°C , 45°C), pH concentrations (3-9), NaCl concentration (1.0%-6.0%), Carbon sources 1.0% (glucose, fructose, lactose, sucrose, maltose, glycerol, mannitol, cellulose and starch) and nitrogen sources 0.5% (sodium nitrate, ammonium nitrate, ammonium sulphate, peptone, beef extract, urea, casein and malt extract) were chosen for growth and antibacterial metabolite production. The growth of the isolate was determined by measuring OD at 540 nm [23].

2.5. Mass production of bioactive metabolite

Mass production of bioactive metabolite was achieved by cultured MRRPK4 in 20 liters of modified nutrient broth. At the end of the incubation, the broth was collected and centrifuged (10000 r/min, 15 min), the supernatant was collected and then fractionated with an equal volume of ethyl acetate. The organic layer was carefully separated and evaporated in a rota evaporator (Buchi, Germany). The extract was dissolved in DMSO and stored.

2.6. Purification of bioactive metabolite

2.6.1. Thin Layer Chromatography (TLC)

TLC of the extract was carried out on TLC analysis plates, 20µl of the extract was loaded on the TLC (Silica gel 60) plates (Merck, Germany). Chloroform: methanol (3:1, v:v) was used for the separation of the compounds.

UV-trans-illumination of the TLC plate was observed at 365 nm [24].

2.6.2. Column Chromatography

Column chromatography was opted for the separation of pure compound. The sample was loaded into a column pre packed with silica gel (Hi-media 100-200 mesh) and eluted by the same solvent used in TLC. The purified fractions were collected and stored for further research [25].

2.7. Structural elucidation of active compound

Fourier Transform Infrared Spectroscopy of compounds defines the existence of different functional group. FT-IR spectroscopy analysis of pure compound is scanned with the frequency range between 4000cm^{-1} and 500cm^{-1}, by using Alpha-T Bruker Spectrometer. Liquid chromatography and mass spectra was carried out in Apex mass spectrum in the negative ion measurement mode with the detection voltage of 1.6kv, and APCI temperature of 400°C , a curved dissolution line of 250°C and the block temperature of 200°C . 1H NMR spectra of compounds was recorded by Bruker DRX 500.13 MHz spectrometer and acquired on a Bruker Avance-2 model spectrophotometer [25].

2.8. Biological activity

2.8.1. Anti cancer activity MTT Assay

MCF-7 cell lines were cultured in Dulbecco's Modified Eagle Medium enhanced with 10% FBS and 0.5 mL^{-1} of penicillin/streptomycin, at 5% CO_2/95% air and 37°C . Doxorubicin was chosen as standard. 96 well plates were used and added 100µL of cell suspension in each well at a density of 10,000 cells per well and incubated for 24h. Cells were then exposed to different concen-trations (10 to 200µg/ml) of the test compounds for 48 h. 10µl of MTT solution (5 mg/ml in PBS) was added to each well (90 µl of the media) and incubated at 37°C for 4h. 200µl of DMSO was added to each well after incubation. OD of solubilized crystals in DMSO was measured at 570 nm on a microplate reader [26]. The percentage growth inhibition was calculated using the formula:

% inhibition = 100 (control-treatment)/control

2.9. Molecular docking studies

2.9.1. Protein and ligand preparation

Crystallographic protein structures of HER2 protein (Pub med ID- 3PP0) were retrieved from protein data bank (PDB). Identification of the active site of these proteins were done by using auto ligand and visualized using the molecular graphics program PyMol® software. Macromolecule which is downloaded in the PDB format was energy minimized using Swiss PDB version 1.4.2.

2.9.2. Ligand preparation

The purified compound (Pyrazine derivative) which was extracted, purified, and spectrally characterized from marine bacterium *Bacillus subtilis inaquosorum* MRRPK4 were sketched using marvin sketch software and the structure of the standard drug doxorubicin was downloaded from drug bank. Both the drug structures were generated in PDB file and their three dimensional structures were visualized using PyMol® software.

2.9.3. Docking studies

Macromolecule and ligand interactions were determined by molecular graphics laboratory (MGL) tools of auto dock version 4.2 and PDB files were converted to PDBQT files. The pre calculated grid maps at the size set at 60, 60, and 60 A° (x, y, and z) to include all the amino acid residues that present in the receptor were done with the generated PDBQT files of macro molecule and ligand [27]. Using 0.375 angstroms as a grid space between points, lamarckian genetic algorithm (LGA) used to chosen and confirm a maximum of 10 conformers required for the docking process with 150 individuals as the population [28, 29]. Default settings of the auto dock were used for rest of parameters. At the end of docking, the best poses were analyzed for hydrogen bonding/π-π interactions and root mean square (RMS) calculations using PyMol® software. Intel CORE™ i3, 64 bit Operating System and 4GB RAM in DELL Windows 10 Laptop were used to complete the docking process.

3. RESULTS

3.1. Isolation and identification of marine bacteria

Out of 10 distinct bacterial strains isolated from Nijampatanam marine sediments (Named as MRRPK1 to 10) which were screened for antibacterial activity, MRRPK4 shows considerable amount of antibacterial activity against *E.coli* (fig. 1) and selected as potential organism which is molecularly identified as *Bacillus subtilis inaquosorum* and deposited in GEN BANK, NCBI as *Bacillus subtilis sb sp inaquosorum* MRRPK4 with Gen Bank accession No. MT421945 (fig. 2).

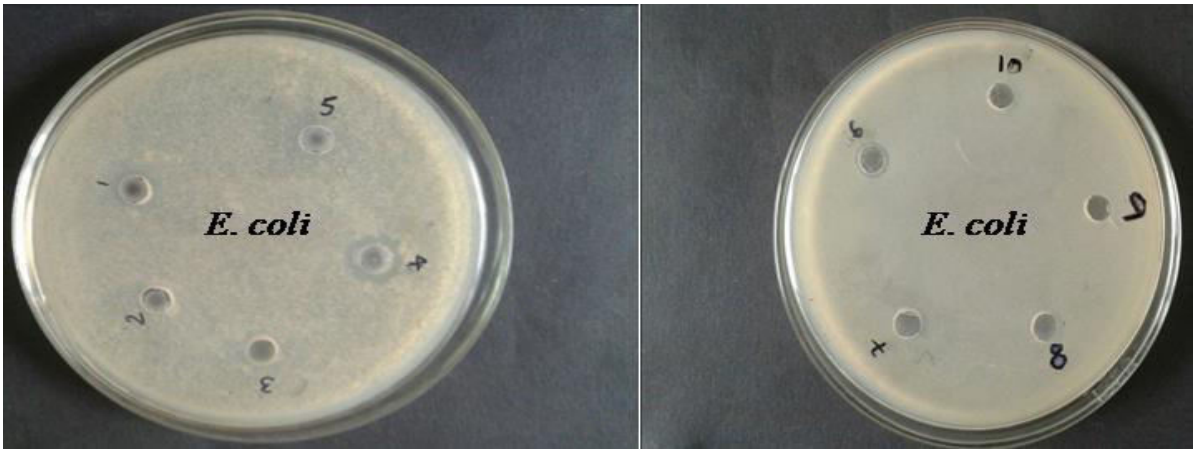


Fig. 1: Screening of antibacterial activity of marine bacteria

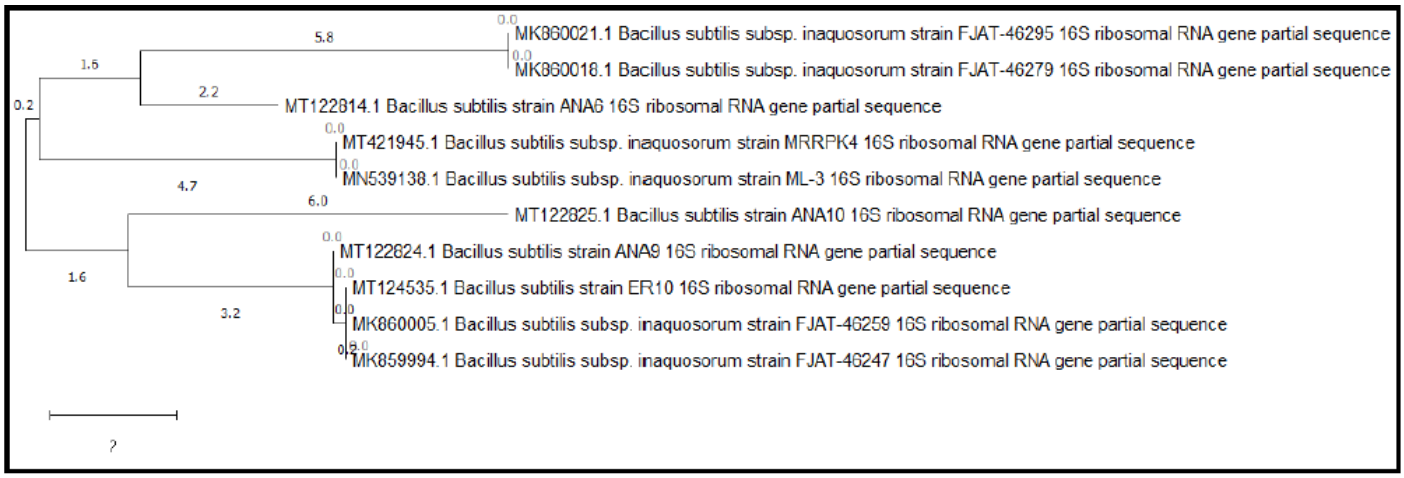


Fig. 2: Phylogenetic analysis of marine bacteria *Bacillus subtilis sb sp inaquosorum* MRRPK4

3.2. Optimization of bioactive metabolite production

The isolate MRRPK4 has shown prominent growth and antibacterial activity when incubated for 60 hrs. 35°C has been chosen for the effectiveness of the isolate with pH 7 and NaCl concentration 5.0%. 1.0% fructose and 0.5% peptone have shown excellent growth of the isolate.

3.3. Thin Layer and column Chromatography

Clear separation of the single compound and purity of the extraction was analyzed by thin layer chromatography. Single spot of compound was resolved with Rf value of 0.68 (Fig. 3).

3.4. Spectral characterization of purified compounds

Mass spectral analysis of purified compound from *Bacillus subtilis sb sp inaquosorum* showed major peak at 6.304 min with molecular weight of 238.29 g/mol, Mass analysis suggested that the compound belongs to pyrazine group with molecular formula $C_{13}H_{22}N_2O_2$ when compared to LC-MS standard from NIST library Fig. 4.

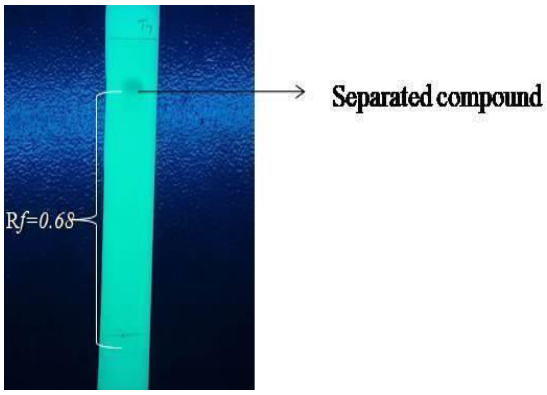


Fig. 3: Thin layer chromatogram showing separated compound

Pure compound from isolate *Bacillus subtilis sb sp inaquosorum* showed the stretch at 2561 indicated the presence of (O-H) hydroxyl group, 3341 N- H group, a stretch at 1709 indicated the C=O group. The result obtained showed the presence of nitrogen containing amine, amide and pyrazine functional groups 3-(3-methylbutan-2-yl) octahydro-pyrrolo(1,2-a) pyrazine-1, 4, dione with a molecular formula C13H22N2O2 (fig.5).

1H NMR spectrum of purified compound from MRRPK4 shows the triplet at d 0.87 due to a terminal methyl group, the strong singlet at d 1.46 is due to long chain methylene groups. The strong signals at d 1.87, 1.96 and 2.17 are due to methylene protons attached to unsaturated systems and the signal at d 3046 are due to two bis allylic protons. The signals at 4.43 (2H) and at 4.60 (1H) suggest that the compound contains two double bonds. Based on spectral charac-teristics of ^{1}H spectrum and database of NIST, was identified as 3-(3-methylbutan-2-yl) octahydropyrrolo (1,2-a)pyrazine-1, 4, dione (fig. 6).

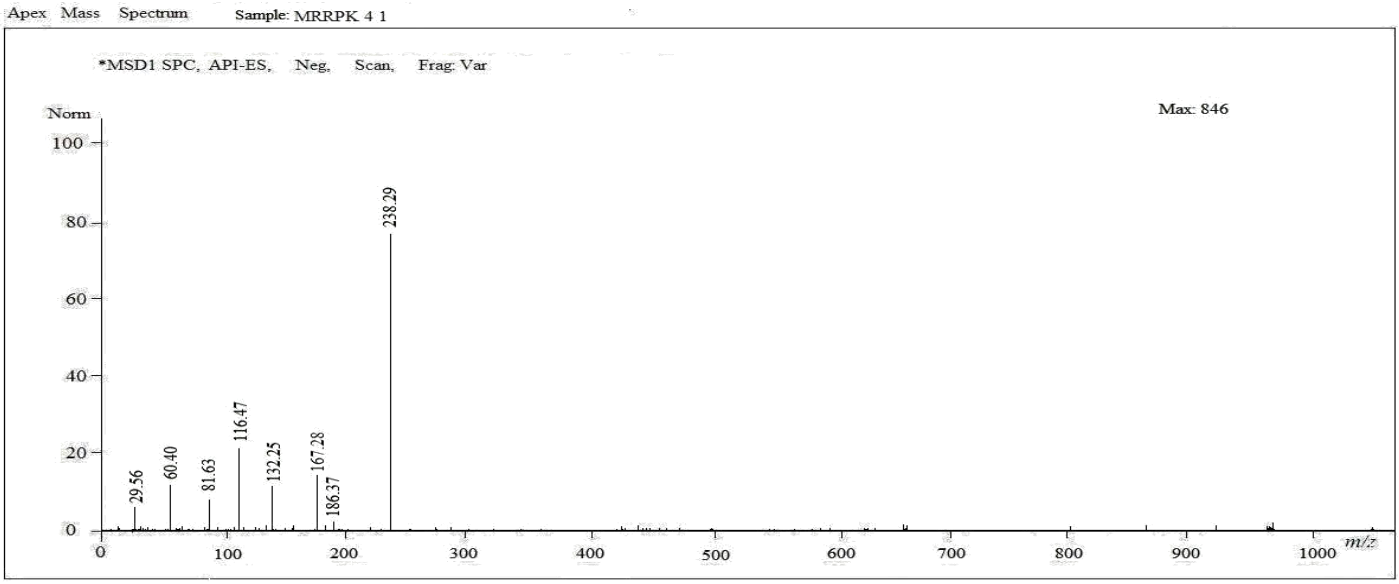


Fig. 4: LC-MS chromatogram of MRRPK4 shows the isolated major peak at 238.29m/z

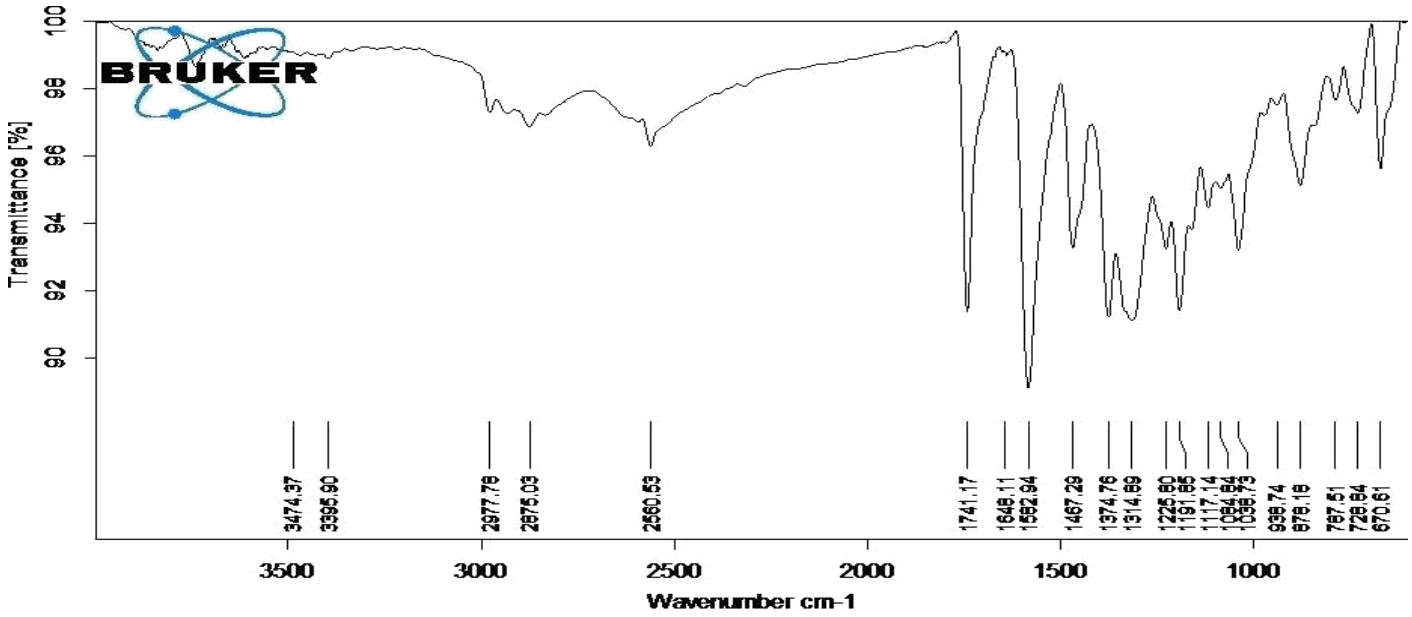


Fig. 5: FT-IR Spectrum showing functional groups of purified compound from MRRPK 4

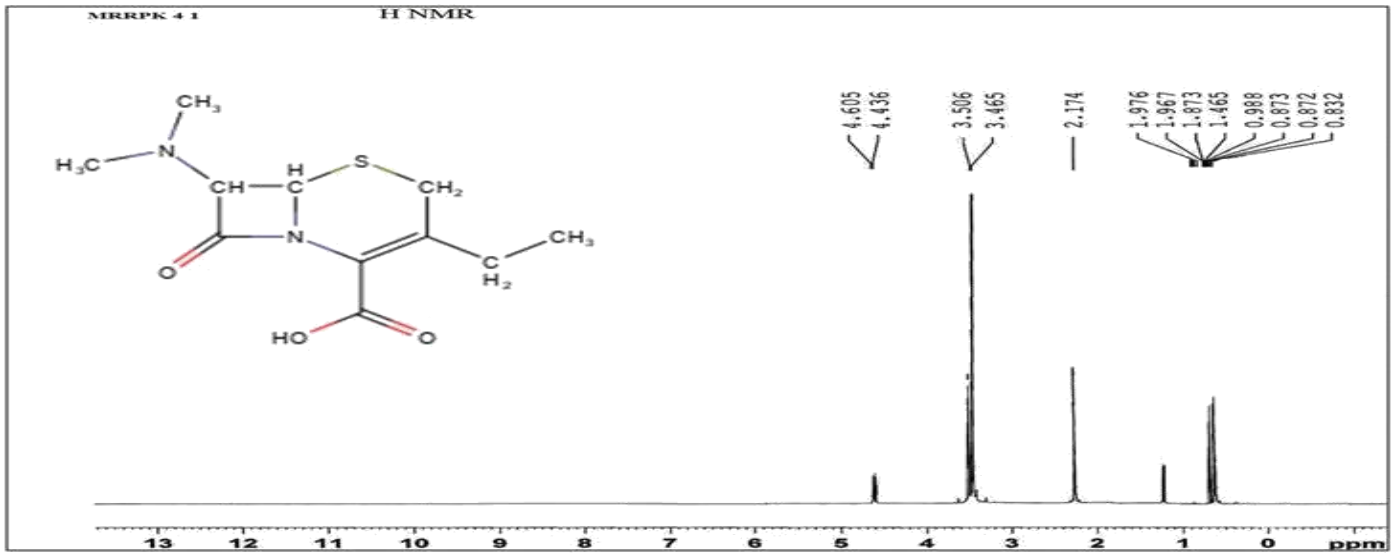


Fig. 6: NMR Spectra of purified compound isolated from MRRPK4

3.5. Biological activity

3.5.1. Anti cancer activity-MTT Assay

Anticancer activity of the pure compound showed considerable effect on MCF-7 cell line. MCF-7cells were treated with 3-(3-methylbutan-2-yl) octahydro-pyrrolo (1,2-a) pyrazine-1,4, dione at the concentration of 100µg/ml, MCF-7 cell viability was decreased to 100% at IC50 value of 12.36µg/ml (table 1).

3.5.2. Macro molecules, ligands and binding sites identification

Three-dimensional structures of breast cancer protein (HER2 protein (Pub med ID-3PP0) and standard anticancer drug doxorubicin (DB00997) were retrieved from protein data bank (PDB) and drug bank (DB) (Fig. 7). The two dimensional structure of spectrally characterized and sketched purified pyrazine derivative was visualized under Pymol as shown in fig 7. The binding (GLY776, GLY778, THR835, VAL777, MET801, THR862, SER783 and THR798) sites of macromolecule for target ligand were determined by using auto ligand, uniport and clustal.

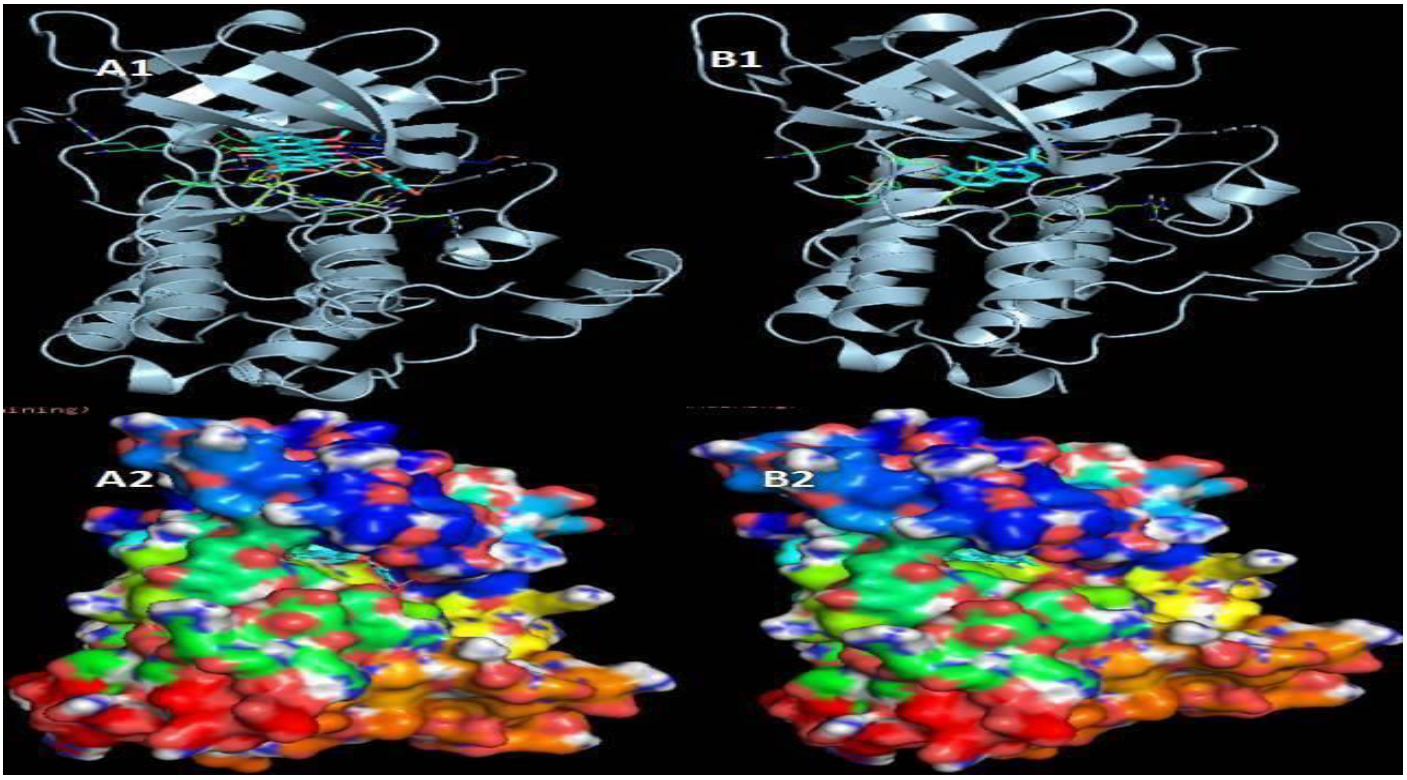
3.5.2.1. Docking studies

Doxorubicin (standard) has docked well at the active site of macro molecule and forms two hydrogen bonds with two amino acids having binding energy of -9.03 and inhibition constant of 241.84nM. Oxygen atom of the ligand forms one hydrogen bond with oxygen atom of ASN850 with bond length of 2.75Å and oxygen atom at sixth position of ligand forms on hydrogen bonds with neighboring nitrogen atoms of LYS753 with the bond lengths of 2.83 Å (fig. 8A).

Purified compound has docked well at the active site of macro molecule with two hydrogen bonds with two aminoacids having binding energy of -6.85 and inhibition constant 958nM. Many of the residues are in close proximity to the compound and are hydrophobic in nature. Oxygen atom at the first position of ligand forms one hydrogen bond with neighboring nitrogen atom of MET801 with the bond length of 2.87 Å. Oxygen atom at third position of ligand forms hydrogen bond with the neighboring oxygen atom of THR862 with the bond length of 3.15 Å (fig. 8B).

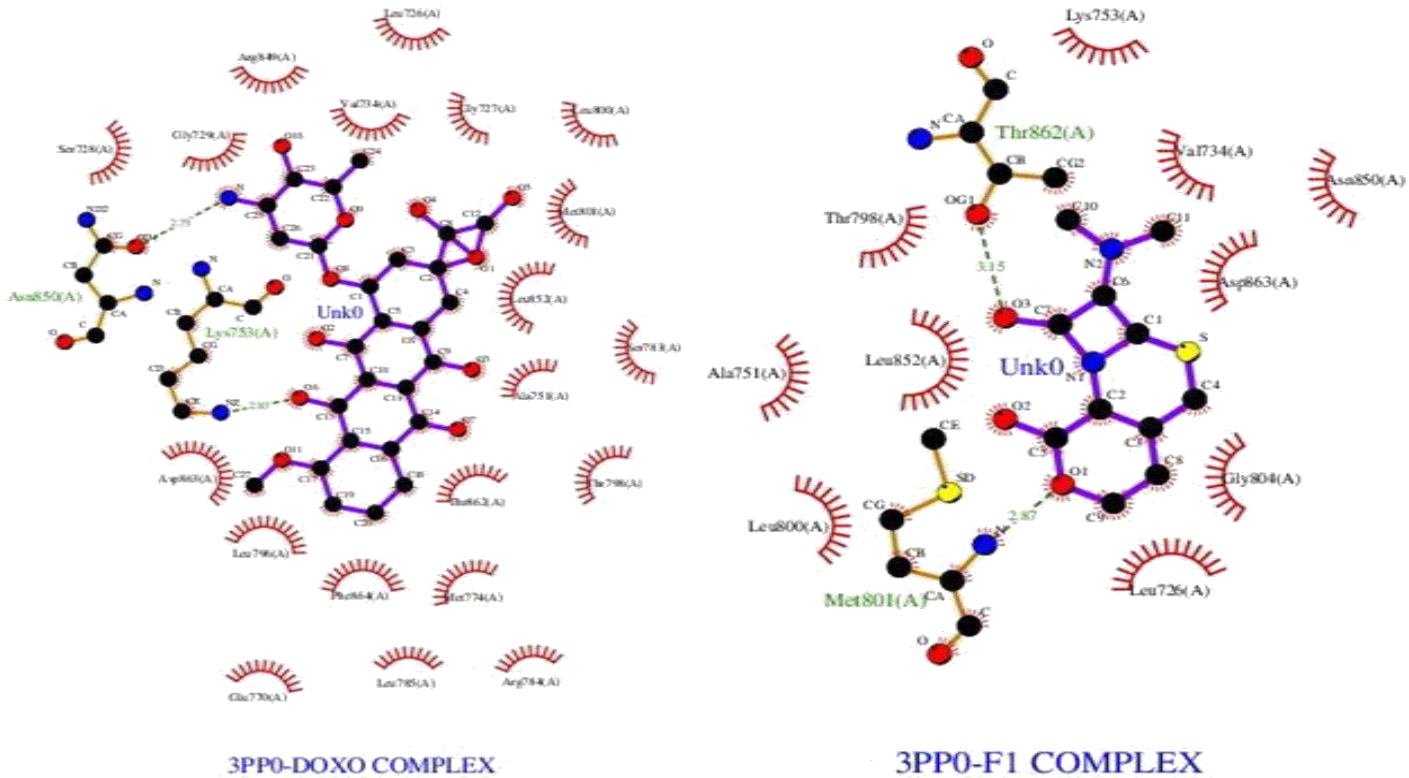
Table 1: Anticancer activity of purified compound from MRRPK4 against MCF-7 cell lines

Concentration (µg)	Absorbance at 570nm	% Inhibition	% Viability	IC50 (µg)
5	0.189	19.91	80.09	12.32
10	0.168	28.81	71.19	
25	0.329	100	0	
50	0.37	100	0	
100	0.382	100	0	
Untreated	0.236	0	100	
Blank	0	0	0	



A1) 3PP0-Doxorubicin structural interaction; A2) best binding pose of 3PP0-Doxorubicin; B1) 3PP0- Purified compound interaction; B2) best binding pose of 3PP0- Purified compound

Fig. 7: Interaction and Binding poses of ligands with 3PP0 protein of MCF-7 cell line



Black- carbon atom; blue- nitrogen atom; red- oxygen atom; Green dotted line- hydrogen bond; red spikes- hydrophobic interactions.

Fig. 8: Ligplot showing the interaction of doxorubicin A and purified compound B

4. DISCUSSION

The isolation of bioactive compounds from bacteria of marine sources, has been a subject of increasing interest in recent years, particularly due to their potential applications in biopharmaceutical and biotechnological domains. Few publications are devoted to the study of the *Bacillus* species isolated from the marine environment. Species of *B. marinus, B. badius B. subtilis, B. cereus, B. licheniformis, B. firmus,* and *B. lentus* were often isolated from marine habitats [30-32]. The present study is an evidence that *Bacillus subtilis sb sp inaquosorum* isolated from marine sediments could be able to produce antibacterial and anti cancer compounds.

Optimization of physico-chemical parameters is an important aspect to be considered in the development of fermentation technology. Formulation of a media that is cost effective for the production of bioactive compounds. Optimization of different physico-chemical parameters for growth and antibacterial activity of *Bacillus subtilis sb sp inaquosorum* was analyzed in order to achieve maximum growth and activity. Okanlawon *et al.*, reported that on the effect of different incubation periods, *B. cereus* recorded the highest growth at 48h. In our study, results showed *Bacillus subtilis sb sp inaquosorum* has shown maximum growth and antibacterial activity at 60h incubation at 35°C with pH 7 and NaCl concentration 5.0% and fructose 1.0%.

The parent ion at a molecular weight of 238 was observed confirms that the molecular weight of compound was found to be 238. Based on the functional groups observed in IR spectra, there is a probability of presence of two amide groups confirms two oxygen's and two nitrogen's in the structure. Carboxylic acids and phenolic structure not observed in IR spectra, confirms the absence of these groups. Hence, the molecular structure of the compound was found to be $C_{13}H_{22}N_2O_2$, with IUPAC name 3-(3- methylbutan-2-yl) octahydro-pyrrolo (1,2-a) pyrazine-1,4,dione.

FT IR results indicated the presence of (O-H) hydroxyl group, C- H group, C=O group (fig. 5). Nuclear Magnetic Resonance Spectroscopy (1H NMR) of the purified compound confirmed the compound as 3-(3-methylbutan-2-yl)octahydropyrrolo(1,2-pyrazine-1,4,dione (FIG. 6). Similar results were obtained by scientists [33] for (9Z,12Z)-octadeca- 9,12-dienoic acid isolated from ethanolic extract of *Cayratia trifolia* (L.) A group of researchers [34] reported that (9Z,12Z)-octadeca-9,12-dienoic acid isolated from the leaves of *Helichrysum pedunculatum* inhibited the growth of different bacteria. Linolenic acid from *Mallotus japonicus* Muell was identified as the antimicrobial substance [35]. Research carried by Kim *et al.,* also showed that t10, c12-CLA increased an apoptosis in human colorectal cancer cells. The anti proliferative effects of Conjugated organic acid on cancer cell lines were supported by the studies [36], which showed a dose-dependent reduction in the proliferation of three different human lung carcinoma cell lines (A-427, SK-LU-1, and A549). Previous studies [37-38] indicated that secondary metabolites decrease the growth of various human cancer cells [39]. Unduri ND also revealed the tumouricidal and antiangiogenic actions of bacterial derivatives. Gamma linolenate isolated from *Spirulina platensis* showed potent cytotoxicity against A-549 cell lines. Our studies also showed the antiproliferation activity in dose dependent manner on MCF-7 cell line with IC_{50} of 12.36µg/ml on MCF-7 cell lines (table 1). the docking studies carried out by Vasanthi, *et al.*, states that the compounds BDBC and MDNP has docked well at the active site of target protein with the glide score of -8.228, glide energy of -55.582 Kcal/mol and -7.952 and glide energy of -40.528 Kcal/mol. Similarly in our study, the bacterial derived organic acid shows almost similar results when compared with the standard drug doxorubicin. Purified compound forms equal number of hydrogen bonds (doxorubicin 2; purified compound), interacting amino acids (doxo-rubicin 6; purified compound 2) and inhibition constant (doxorubicin 241.84nM; purified compound 954nM) this is because of its purity and specificity of the standard (fig. 8).

5. CONCLUSION

In this research, the novel isolated and identified pyrazine compound from *Bacillus subtilis sb sp inaquosorum* showed a wide range of cytotoxic activity against MCF-7 cell lines with IC_{50} of 12.32µg/ml. The compound also exhibited potential results *in silico* when compared with the standard drug doxorubicin. From this study we could consider the bacterial derived secondary metabolites as a potential source for the development as an antibacterial and strong anticancer agent for controlling diseases.

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Conflict of interest

Authors declare no conflict of interest.

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