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PURIFICATION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF PROTEINS FROM MARINE BACTERIUM

S. Shanthasubitha*[1], S. Saravanababu[2] and B. Shanmugapriya[2],
[1]*Biiogenic, Institute of Biotechnology, Namakkal – 637 001, Tamil Nadu, India.*
[2]*C. N. College, Erode, Tamil Nadu, India.*

Abstract

The marine water samples were collected from the Kilakarai deep sea, the bacterial colonies were isolated and the dominated colonies were picked out and their morphological characterization was studied. From the biochemical characterization the isolated colony results as *Bacillus* sp. The antimicrobial potential were analysed and the resulted Fraction I, Fraction II, Fraction III shows the greater effect when against with the clinical pathogens. *Staphylococcus aureus, Pseudomonas* sp., Fraction III shows greater zone of inhibition in various concentration of (25 µl, 50 µl, 75 µl and 100 µl). The higher concentration of 100 µl of sample shows the greater results. The high concentration of the 100 µl of the crude sample exhibit higher zone of inhibition against the *Staphylococcus* sp. (14 mm) and *Pseudomonas* sp. (13 mm). The results suggested that the Fraction III warranted further purification in order to unveil the active components of *Pseudomonas* sp. From the curve of elution in DEAE Sepharose Fast Flow chromatography, three A280 nm peak fractions, named G-1, G-2, G-3 were collected respectively. The antimicrobial potential of the marine microbes the G-1, G-2, G-3 shows the greater effect when against with the clinical pathogens *Staphylococcus aureus* and *Pseudomonas* sp. Among that G-3 shows the maximum results of 18.2 mm and 15.4 mm against *Staphylococcus aureus* and *Pseudomonas* sp. respectively. The present study crude extract of marine *Bacillus* sp. showed anticancer activity against HT-15 and HT-29 cell line in a dose dependant manner. The crude extract of marine *Bacillus* sp. showed activity of 7.88 at 400 µg/ml. Whereas, the purified crude of Fraction I, Fraction II and Fraction III showed 7.99 %, 20.67 % and 29.30 % of inhibition at the same concentration respectively against HT-15 and the purified crude of Fraction I, Fraction II and Fraction III showed 8.00 %, 9.12 % and13.02 % of inhibition at the same concentration respectively against HT-29. The purified sample of G1, G2 and G3showed 18.63 %, 15.80 % and 76.31 % of inhibition at the same concentration respectively against HT-15 and the purified sample of G1, G2 and G3 showed 12.90 %, 12.53 % and 69.32 % of inhibition at the same concentration respectively against HT-29. Estimation of amino acids was done using an automatic amino acid analyzer for the two fractions of FIII and G3.

Key words: Protein, *Bacillus* sp., *Pseudomonas* sp. and Antimicrobial activity.

1. Introduction

The world's oceans, covering more than 70 % of the earth's surface, represent an enormous resource for the discovery of potential chemotherapeutic agents. Taking higher Taxonomic levels as an estimate of biodiversity, more phyla are found in the oceans than on land. Of the thirty three known phyla of extant animals, only one is exclusive of land, while as many as twenty-one phyla are exclusive of the sea. Marine biotechnology is the science in which marine organisms are used in full or partially to make or

*Corresponding author: **S. Shanthasubitha**
E-mail: subithabio@gmail.com
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modify products, to improve plants or animals or to develop microorganisms for specific uses. With the help of different molecular and biotechnological techniques, humans have been able to elucidate many biological methods applicable to both aquatic and terrestrial organisms. According to (Harvey, 2000), only 10 % of over 25,000 plants have been investigated for biological activity.

Bacteria and fungi are prime producers of the antagonistic substances in terrestrial environment. A similar role in the oceans from these organisms was expected. Indeed, this had been found true. Antibiotic, antiviral, antifungal, and antiyeast activities of these organisms had been reported. Besides, a few growth stimulant properties which may be useful in studies on wound healing, carcinogenic properties and in the study of cancers are reported. Among the many bacteria showing antimicrobial activity, a variant of the ichthyotoxic Pseudomonas piscicida, exhibited marked antagonism to various microorganisms (Buck *et al*., 1962). As the study of marine bacteria and their potential role in the production of pharmacologically active metabolites is a promising new topic for research, in the present study we isolated and characterized bacteria with antimicrobial activities from clinical pathogens

2. Materials and methods

Collection of sample

The marine water samples were collected from Kilakarai the deep sea.

Isolation of bacterial strains from the samples

The bacterial colony were isolated by Serial dilution method and identified by standard methods.

Extraction of total proteins

Marine bacteria isolated as above were cultured in 300 ml Marine $FePO_4$. An amount of 0.1 g was dissolved in 1 l seawater with pH 7.2 – 7.6 for the production of bioactive compounds in 500 ml Erlenmeyer flasks and incubated on a Rotatory shaker at 220 rpm at 25 °C. After 7 days of cultivation, the cultures were centrifuged. After centrifugation (16,000 g, 20 min) at 4 °C, the supernatant was collected and the crude extract obtained. The crude extract of bacteria was fractionated by salting out with increasing concentrations of ammonium sulfate. Solid ammonium sulfate was slowly added to the above crude extract with gentle stirring, upto 35 % saturation in 20 min. After the crude extract was left at 4 °C with the ammonium sulfate under vortexing for another 40 min, the protein precipitate was collected by centrifugation (16,000 g, 20 min) at 4 °C. The supernatant was transferred to another beaker, and solid ammonium sulfate was added to it upto 70 % saturation. The mixture was treated as above. Likewise, another protein precipitate was obtained at 70 % − 100 % saturation of ammonium sulfate. Each of the three protein pellets was suspended in 10 ml of ice-cold PBS (10 mM, pH 8.0), and dialyzed against a large volume (3 L) of distilled water for 24 hrs at 4 °C. Dialysis bags were employed. During this process, the dialysate was changed three times to completely remove any residual ammonium sulfate (Murphy *et al*., 1990).

Antimicrobial Activity by Agar Well Diffusion Assay (Perez *et al.*, 1990)

The agar well diffusion method was used for the inhibitory effects of crude extract of marine *Bacillus* against the clinical pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* extract was loaded on the Mueller Hinton agar plates. This was swabbed with clinical pathogen such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Five wells (6 mm in diameter) were made equidistance in each of the plates using a sterile cork borer. Upto 25 µl to 100 µl of each concentration of the extract were respectively introduced into the wells using sterile automatic pipettes, with the stock in one well. It was allowed to diffuse at room temperature for 2 hrs and the plates were incubated to 37 °C for 24 hrs. Diameters of the inhibition zones were measured. The antibacterial activity was expressed

as the mean zone of inhibition diameters (mm) produced by marine microbes.

Anticancer activity – Cytotoxicity study

Human cell lines

HT-29 and HT-15 human cancer cell lines of colon grown in RPMI medium were obtained from CMC hospital, Vellore.

***In vitro* cytotoxicity studies (Sini *et al.*, 2012)**

The crude extract of marine *Bacillus* was studied for short term *in vitro* cytotoxicity using HT-15 HT-29.10mg of the extract was taken in an Eppendorf vial of capacity 1 ml and dilute to four different concentrations with its duplicate and control (50 %) using solvent and mixed with the help of a vortexing machine. The cell viability was checked by trypan blue dye (1 %). The cell suspension (1×10^6 cells in 0.1 ml) was added to tubes containing various concentrations of the test compounds and the volume was made upto 1 ml using phosphate buffered saline (PBS). Control tube contained only cell suspension. These assay mixtures were incubated for 3 hour at 37 ºC. After incubation, 0.1 ml trypan blue was added and number of dead cells determined by using haemocytometer. The percent viability was calculated by using formula:

% viability = live cell count/total cell count × 100

Micro culture tetrazolium (MTT) assay

Cell viability was assessed by MTT assay (Micro culture tetrazolium/formazan assay) in the presence and absence of different concentrations of the plants extract. The cells were seeded in 96 well plates. Four wells for each concentration were seeded and triplicate plates were used the cell line. Then, the cells were incubated at 37 °C. After 24 hrs, the medium was replaced by fresh medium containing different concentrations of the plants extract. Then, the medium was changed by fresh medium containing MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 4-diphenyltetrazolium bromide] with a final concentration of 0.5 mg/ml (after 24 hrs). The cells were incubated for another 4 hrs in a humidified atmosphere at 37 °C and after that the medium containing MTT was removed and remaining MTT formazan crystals were dissolved in DMSO. The absorbance was measured at 570 nm immediately using an ELISA reader. IC_{50} was defined as the concentration of the extract that produced a 50 % decrease in cell viability relative to the negative control which was wells exposed to the solvent without any extract.

Purification of proteins by Anion exchange chromatography (DEAE Sepharose Fast Flow)

The Fraction - I, Fraction - II and Fraction - III were obtained at the ammonium sulfate saturation of 0 – 35 %, 35 – 70 % and 70 – 100 %, Fraction III were active in the antimicrobial activity. Fraction III were obtained from the crude extract at 70 % − 100 % saturation of ammonium sulfate was dialyzed against 10 mM Tris - HCl, pH 7.46 for 5 hrs and the dialyzed solution was subsequently injected into a DEAE Sepharose Fast Flow column, which was pre-equilibrated with the aforementioned Tris-HCl buffer. The column was washed with the same buffer until the baseline returned to zero and remained stable. The column was then eluted with increasing concentration of NaCl prepared in 10 mM Tris-HCl buffer, pH 7.46 at 4 °C. Aliquots of 5 ml/tube were collected at a flow rate of 1.2 ml/min and the absorbance was measured at 280 nm. Seven A280 nm peak fractions, named G-1, G-2, and G-3 were collected respectively.

Protein assay

The protein content of total protein extract Fraction - III, G-1, G-2 and G-3 was determined by Lowry's method

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

The Sodium dodecyl sulphate - polyacrylamide gel electrophoriesis (SDS-PAGE) analysis was performed (Fraction III and G1).

Analysis of amino acid component

The qualitative and quantitative estimation of amino acids was done using an automatic amino acid analyzer (Shimatzu-High performance

liquid chromatography LC 4A). Twenty µl of the purified sample was injected into single column and analyzed using sodium buffer system (Yamamoto *et al.*, 1994).

3. Results

The marine water samples were collected from the Kanyakumari deep sea, and the sample was transferred to the lab aseptically, the collected sample was stored in the air tight container. The water sample was serially diluted and spreaded into the marine agar medium. After incubation period the colonies were counted and the dominated colonies were picked out and their morphology morphological characterization was studied. The dominated colony was streaked and the pure culture was stored for further studies .The isolated colony results as *Pseudomonas* sp.

The Fraction - I, Fraction - II and Fraction - III were obtained at the ammonium sulfate saturation of 0 – 35 %, 35 – 70 % and70 – 100 %. The antimicrobial potential of the marine microbes the Fraction - I, Fraction – II and Fraction - III shows the greater effect when against with the clinical pathogens. *Staphylococcus aureus* and *Pseudomonas* sp. Fraction - III shows greater zone of inhibition in various concentration of (25 µl, 50 µl, 75 µl and 100 µl). The higher concentration of 100 µl of sample shows the greater results. The high concentration of the 100 µl of the crude sample exhibit higher zone of inhibition against the *Staphylococcus* sp. (14 mm) and *Pseudomonas* sp. (13 mm) which was shown in the Table - 3. The results suggested that the Fraction - III warranted further purification in order to unveil the active components of *Pseudomonas* sp.

Column specification: 1.6 × 30 cm; Equilibrate liquid: buffer C (Tris-HCl, pH 7.46, 10 mM); Sample: Fraction-III; Detection wavelength: UV 280 nm; Flow rate: 1.2 ml/min; Collection rate: 5 ml/tube. Aliquots of 5 ml/tube were collected at a flow rate of 1.2 ml/min and the absorbance was measured at 280 nm. Three A280 nm peak fractions, named G-1, G-2, G-3 were collected respectively.

The present study crude extract of marine *Bacillus* showed anticancer activity against HT-15and HT-29 cell line in a dose dependant manner. The crude extract of marine *Bacillus* sp. showed activity of 7.88 at 400 µg/ml. Whereas, the purified crude of Fraction I, Fraction II and Fraction III showed 7.99 %, 20.67 % and 29.30 % of inhibition at the same concentration respectively against HT-15 and the purified crude of Fraction I, Fraction II and Fraction III showed 8.0 %, 9.12 % and 13.02 % of inhibition at the same concentration respectively against HT-29. The purified sample of G1, G2 and G3 showed 18.63 % , 15.80 % and 76.31 % of inhibition at the same concentration respectively against HT-15 and the purified sample of G1, G2 and G3 showed 12.90 % , 12.53 % and 69.32 % of inhibition at the same concentration respectively against HT-29. The molecular weight of G-3 and Molecular weight marker was ranged from 14.4 kDa to 97.4 kDa. Three bands appear, the molecular weight of band 46 kDa, band 35 kDa and band 32 kDa. Estimation of amino acids was done using an automatic amino acid analyzer for the two fractions of F - III and G3. The amino acids present in these two samples were shown in the table.

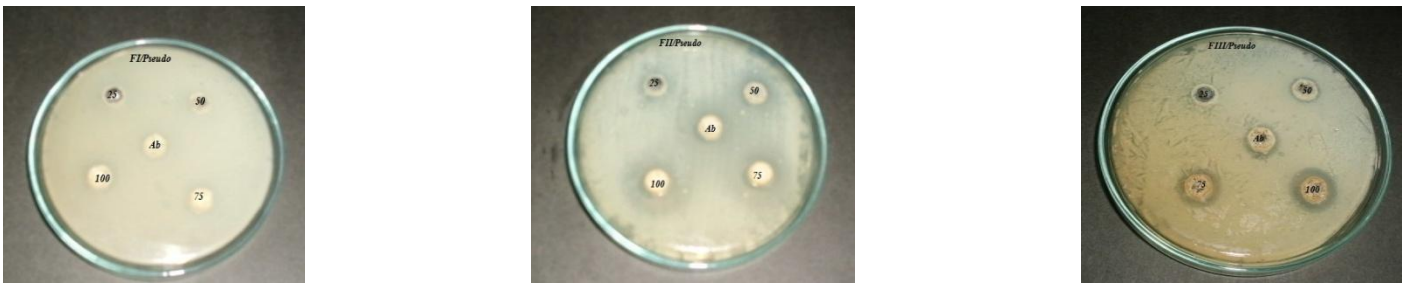


Figure - 1: Antimicrobial activity of the crude extracts (Fraction I, Fraction II. Fraction III) against *Pseudomonas aeruginosa*

Figure - 2: Antimicrobial activity of the crude extracts (Fraction I, Fraction II. Fraction III) against *Staphylococcus aureus*

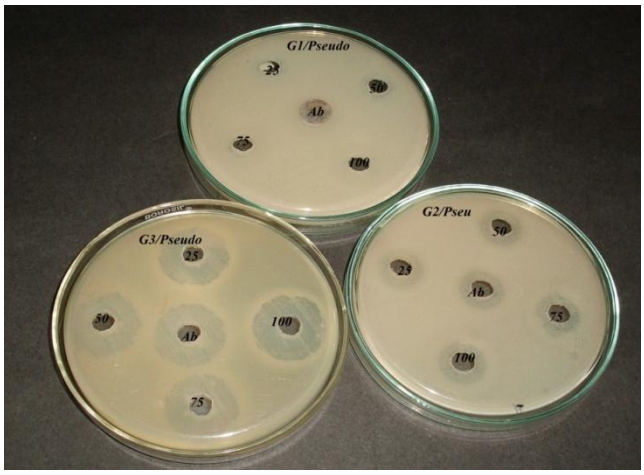
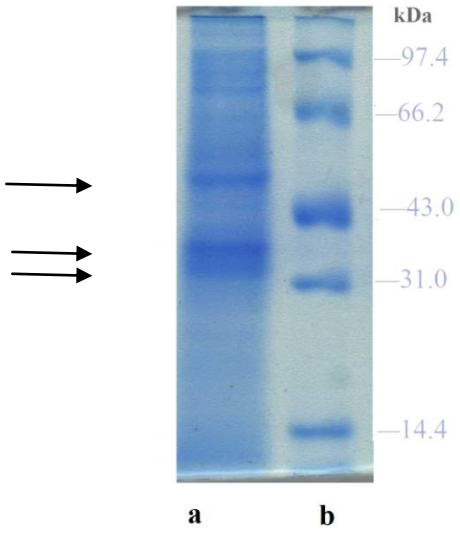


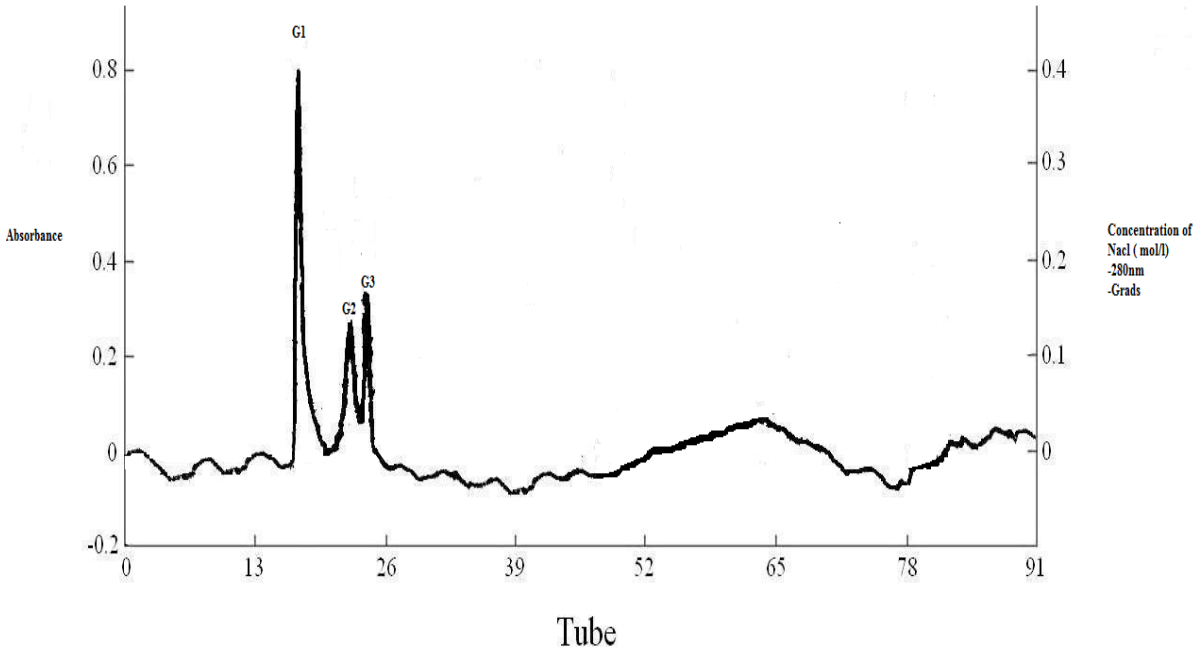
Figure - 3: Antimicrobial activity of the purified sample (G1, G2, and G3) against *Pseudomonas aeruginosa*

Figure - 4: Antimicrobial activity of the purified sample (G1, G2, and G3) against *Staphylococcus aureus*



Molecular weight marker (range from 14.4 kDa to 97.4 kDa). Three bands appear, the molecular weight of band 46 kDa, band 35 kDaand band 32 kDa

Figure - 5: The SDS-PAGE of G3

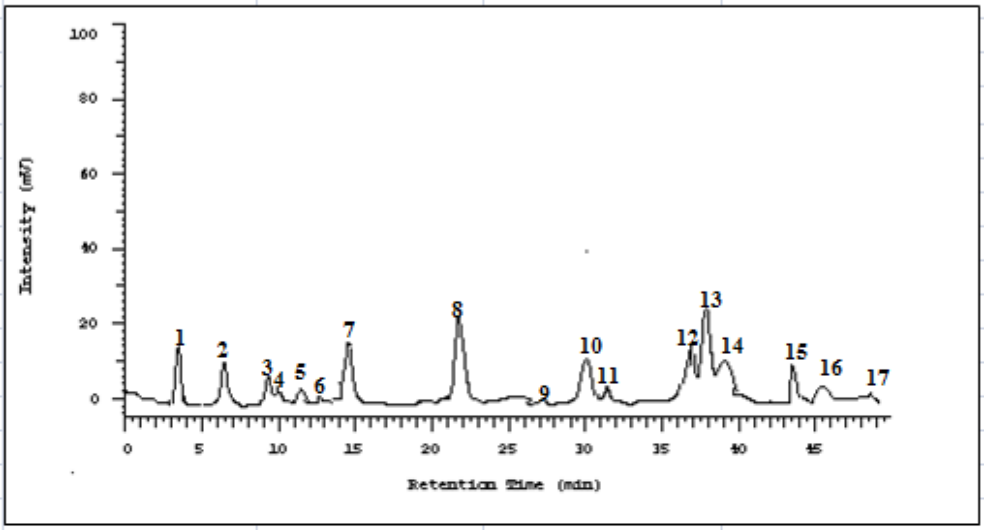


Column specification: 1.6 × 30 cm; Equilibrate liquid: buffer C (Tris-HCl, pH 7.46, 10 mM); Sample: Fraction - III; Detection wavelength: UV 280 nm; Flow rate: 1.2 ml/min; Collection rate: 5 ml/tube

Figure – 6: The curve of elution in DEAE Sepharose Fast Flow chromatography

D-7000 HPLC System Manager Report

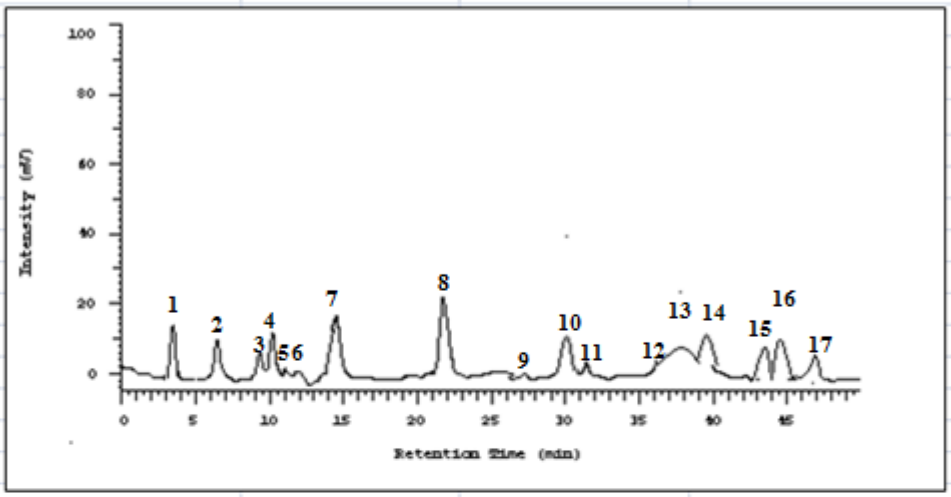
System Name:	**LACHROM L-7000**	**Series:**	**AMINO ACIDS**
Proc. Method:	**Atoz**	**Sample Name:**	**FIII**



No.	COMPONENT NAME	R.T.	AREA	AREA %
1	ASPARTIC ACID	4.06	10123	0.113
2	GLUTAMIC ACID	6.49	454	0.0343
3	ASPARAGINE	9.21	116	0.0011
4	GLYCINE	13.43	147	0.0054
5	THREONINE	14.9	3465	0.201
6	ARGININE	18.09	2604	0.109
7	ALANINE	19.55	134	0.005
8	CYSTINE	21.66	3423	0.2007
9	TYROSINE	24.78	121	0.008
10	HISTIDINE	27.44	324	0.0456
11	VALINE	29.91	1343	0.073
12	ISO-LEUCINE	36.65	343	0.024
13	PHENYL ALANINE	37.95	6545	0.298
14	LEUCINE	39.36	4067	0.189
15	LYSINE	43.41	6232	0.785
16	PROLINE	45.12	121	0.005
17	TRYPTOPHAN	46.13	878	0.078
				2.1850

D-7000 HPLC System Manager Report

System Name:	**LACHROM L-7000**	**Series:**	**AMINO ACIDS**
Proc. Method:	**Atoz**	**Sample Name:**	**G3**



No.	COMPONENT NAME	R.T.	AREA	AREA %
1	ASPARTIC ACID	4.03	129124	9.972
1	GLUTAMIC ACID	6.49	90531	8.35
2	ASPARAGINE	9.24	67525	1.549
3	SERINE	10.22	1301	0.072
4	GLYCINE	13.45	2012	0.201
5	THREONINE	14.89	121210	7.543
6	ARGININE	18.08	119	0.063
7	ALANINE	19.53	115	0.004
8	CYSTINE	21.67	305237	22.100
9	TYROSINE	24.76	115	0.004
10	HISTIDINE	27.25	10741	0.537
11	VALINE	29.90	10521	0.425
12	ISO-LEUCINE	36.62	12012	3.874
13	PHENYL ALANINE	37.93	16530	5.325
14	LEUCINE	39.34	18491	1.370
15	LYSINE	43.39	138124	13.771
16	PROLINE	45.11	209671	20.652
17	TRYPTOPHAN	46.12	475	0.321
			1133866	**96.133**

Figure – 7: Analysis of Amino acids by HPLC

4. Discussion

Marine bacteria have been recognized as important and untapped resource for novel bioactive compounds (Anand *et al*., 2006; Uzair *et al*., 2006). Development of marine biotechnology was expected to produce novel compounds that may contribute significantly towards drug development over the next decade. In this study, we isolated several marine bacteria for extraction of some possible types of antimicrobial compounds active against drug resistant organisms. To demonstrate and characterize this possible antagonistic ability, antibiotic resistant clinical isolates and indigenous marine bacteria were used as revelatory.

In present study the antimicrobial potential of the marine microbes the Fraction I, Fraction II and Fraction III shows the greater effect when against with the clinical pathogens. *Staphylococcus aureus* and *Pseudomonas* sp. Fraction III shows greater zone of inhibition. The results suggested that the Fraction III warranted further purification in order to unveil the active components of *Pseudomonas* sp. From the curve of elution in DEAE Sepharose Fast Flow chromatography, three A280 nm peak fractions named G-1, G-2, G-3 were collected respectively. In the previous studies of the Liyan Song *et al*. (2008) isolated seven fraction from the protein of invertebrates. The antimicrobial potential of the marine microbes the G-1, G-2, G-3 shows the greater effect when against with the clinical pathogens *Staphylococcus aureus* and *Pseudomonas* sp. Among that G-3 shows the maximum results of 18.2 mm and 15.4 mm against *Staphylococcus aureus* and *Pseudomonas* sp. respectively. Estimation of amino acids was done using an automatic amino acid analyzer for the two fractions of FIII and G3. In the previous, Yamamoto *et al.* (1994) studied the amino acids and the results were similar to the present studies. In the present study, the crude extract were partially purified by the ion exchange chromatography and the antimicrobial activity was analysed and the compound was considered as the bioactive compound because of the biopotentiaal of the compound against the clinical pathogen (Kondratieva and Vakhusheva, 1991).

The crude extract from marine *Bacillus* was initially evaluated for their effects on cell viability through cytotoxic test. The cytotoxic effect of crude extract from marine *Bacillus* was observed for 72 hrs of incubation period and the CC_{50} value was calculated as 500 µg/ml. The purified crude of Fraction I, Fraction II and Fraction III showed 7.99 %, 20.67 % and 29.30 % of inhibition at the same concentration respectively against HT-15 and the purified crude of Fraction I, Fraction II and Fraction III showed 8.0 %, 9.12 % and 13.02 % of inhibition at the same concentration respectively against HT-29. The purified sample of G1, G2 and G3 showed 18.63 %, 15.80 % and 76.31 % of inhibition at the same concentration respectively against HT-15 and the purified sample of G1, G2 and G3showed 12.90 %, 12.53 % and 69.32 % of inhibition at the same concentration respectively against HT-29. The IC_{50} value shows the inhibition concentration at which only 50 % of the cells are viable. It was reported that crude materials that require a drug concentration greater than 30 µg ml^{-1} to exhibit cytotoxicity were not considered as cytotoxic agents (Quah, 1995). Molecular weight of the G-3 were analysed with Molecular weight marker (range from 14.4 kDa to 97.4 kDa). Three bands appear, the molecular weight of band 46 kDa, band 35 kDaand band 32 kDa (Liyan Song *et al.*, 2008). Based upon the results of the *in vitro* antimicrobial assay, two pratien samples Fraction III and G3 from *Pseudomonas* sp. shows the bioactivity - guided fractionation and purification. Important characteristics of proteins G3 were identified, showing three bands and the molecular weights to be 46 kDa, band 35 kDa and band 32 kDa. Additionally, our present study reveals that the studies of amino acids pharmaceutical

agents were discovered by screening natural products from marine microorganisms.

5. Reference

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