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Isolation and partial characterization of anti cancer protein from *Lysinibacillus sphaericus* against HEPG2 cancer cell line

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Abstract

Lysinibacillus sphaericus was isolated from soil sample and confirmed by 16s rDNA sequencing. The anti cancer protein was obtained by ammonium sulphate precipitation and purified. The lyophilized fractions were screened for anti MRSA (Methicillin Resistant *Staphylococcus aureus*) and anti cancer activity against liver cancer cells (Hep G2) 150µg/ml of protein exhibited the high level of cytotoxicity (75%) towards the Hep G2 cells where the positive control cyclophospamide showed cytotoxicity of 76%. The anti cancer protein was characterized by peptide Mass Fingerprinting using MALTI TOF MS.

Keywords: Anticancer peptide, Anticancer protein, Antimicrobial peptides.

Introduction

The development of new classes of anticancer drugs that lack the toxicity of conventional chemotherapeutic agents and are unaffected by common mechanism of chemo-resistance would be a major advance in cancer treatment.

Numerous chemotherapeutic drugs have been developed to treat cancers, including DNA alkylating agents, antimetabolites and hormone agonists/ antagonists. Although these drugs have been successfully used for the treatment of metastatic cancers, severe side effects and dose limitations are prevalent. As a result of their inability to distinguish between cancer cells and proliferating normal cells, current drugs kill both. More over cancer cells develop resistance to these drugs that is mediated by the over expression of the multidrug resistance proteins that pump the drug out of the cells and thus render the drugs ineffective [8].

Thus there is an urgent need to develop new classes of anticancer drug with new mode of action that selectively target the cancer cells. Cationic peptides are widely distributed in living organisms playing a variety of functions. They are often referred to as antibacterial or antimicrobial peptides due to their well characterized role in innate immunity against infectious agents. [2]While most of the studies on cationic peptides have focused on their antimicrobial activity, other biological effects are emerging and in the past few years the ability of some peptides to affect the tumor cells has been reported [1,4,7,10].Most of these peptides have an amphipathic structure and they preferentially bind and insert into negatively charged cell membranes, consequently destabilization of the membrane disturbs the lectrolyte balance and induces the intracellular contents to leak leading to cell death. In contrast to normal eukaryotic cells, which generally have low membrane potentials

and whose outer leaflet almost exclusively consist of Zwitter ionic phospholipids, the prokaryotic and cancer cell membranes maintain large transmembrane potentials and have a higher content of anionic phospholipids on their outer leaflet. Many AMP therefore preferentially disrupt prokaryotic and cancer cell membranes rather than eukaryotic membrane[6]. The present study was undertaken to characterize and evaluate the anti cancer potential of peptide isolated from *Lysinibacillus sphaericus* a new classes of anti cancer peptide.

Materials and Methods

Sample Collection

Soil samples from Valparai, Coimbatore district (Tamil Nadu) were collected randomly from different areas of the cultivated land in sterilized poly bags sealed properly and brought to the laboratory for further analysis. The samples were then stored at 4˚C till used.

Isolation of Bacteria

Serial dilution agar plating method (or viable plate counting method) was followed to isolate bacteria from sample. One gram of soil sample was added to 100 ml of sterile distilled water and serially diluted up to 10^{-8} dilution. Following that 0.1mL of each of the dilution was transferred to sterile nutrient agar plates and spread using L-rod. The plates were then incubated at 34˚C for 24 hours to obtain colonies. The individual colonies were chosen upon the basis of their characteristics such as size, shape, surface appearance, texture and color which was then streaked onto fresh nutrient agar plates for purification. This process was repeated several times until pure cultures of the isolates were obtained. The purified colonies were sub cultured on to nutrient agar slants and stored at 4˚C for further studies and screened for its antimicrobial activity.

Screening of Soil Bacteria for Its Antimicrobial Activity Point Inoculation Method

Muller Hinton Agar plates were prepared .The overnight culture of bacterial pathogens in nutrient broth was uniformly swabbed on the surface of Muller Hinton agar plates using sterile cotton swab, making a lawn culture. After drying the agar surface for 5 minutes, soil bacterial isolates from the agar slants were spotted on to Muller Hinton Agar plates seeded with actively growing cells of the clinical pathogens. The plates were incubated at 37˚C for 24 hours. After the incubation period, the plates were observed for the zone of inhibition and the results were recorded. Then the isolates obtained from the soil samples were spotted on to the Muller Hinton Agar plates which are seeded with MRSA. The plates were incubated at 34^0C for 24hours. After the incubation period the plates was observed for the zone of inhibition. The bacterium which showed the antibacterial activity was identified by 16S r DNA sequencing.

Production and Purification of Antimicrobial Protein

For the production of antimicrobial protein the bacterial isolates *Lysinibacillus sphaericus* were grown in 500 ml TSB medium at 37°C in a rotary shaker at 125 cycles per minute for desired times. Determination of the number of viable cells were (CFU ml^{-1}) carried out by viable plate count method. After cultivation for 72 hours the CFS was collected by centrifugation at 10,000 g for 15 minutes. To the cell free supernatant, ammonium sulphate was added to achieve 30% saturation. It was stirred well and incubated at 4°C for overnight. After incubation period, the precipitate was collected by centrifugation at 6000rpm for 15 minutes at 4°C.The precipitates were dissolved in sterile phosphate buffer. Antimicrobial activity of the precipitate was tested using agar well diffusion assay and the results were recorded. The ammonium sulphate precipitates of various percentage of saturation (10-80%) were desalted by dialysis. Pre-treatment of dialysis bag plays an essential role in dialysis. Dialysis membrane was taken and treated with distilled water at 65ºC for 10 minutes to remove glycol and then soaked in 10mM disodium Ethylene Diamine Tetraacetic Acid (EDTA). It was then treated with 10mM Sodium bicarbonate to remove sulphur molecule and finally washed with distilled water. The membrane was clipped at one end and one third of the bag was filled with ammonium sulphate precipitates and the other end was also clipped using dialysis tubing clip. The samples were then dialyzed against phosphate buffer saline (pH 7.4) by changing buffer in every four hours for overnight. Then the dialysis bag was taken and the samples were carefully drawn and collected for further analysis. The inhibitory activity and protein content of 10-80% saturated dialyzed protein were examined and the percentage of saturation which exhibited maximum inhibitory activity was taken for anti cancerous study.

MTT Assay (Anti proliferative assay)

The HepG2 Cancer cells were grown in a 96-well plate in Delbucco's minimum essential medium (DMEM, Hi Media) supplemented with 10% fetal bovine serum (Gibco Laboratories) and antibiotics (Streptomycin, Pencillin-G, Kanamycin, Amphotericin B). About 1.0 ml cell suspension (105 cells/ ml) was seeded in each well and incubated at 37ºC for 48 hours in 5% CO_2 for the formation of confluent monolayer. The monolayer of cells in the plate was exposed to various concentrations of protein obtained from *Lysinibacillus sphaericus*. The cell viability was measured using MTT assay with MTT solution (5 mg/ mL) and DMSO. This tetrazolium salt is metabolically reduced by viable cells to yield a blue insoluble Formosan product measured at 570nm spectrophotometrically. Controls were maintained throughout the experiment (untreated wells as cell control). The assay was performed in triplicate for each of the concentration. The mean of the cell viability values was compared to the control to determine the effect of the protein on cells and percentage of cell viability was plotted against concentration of each of the protein concentration.

Anticancerous activity

MTT assay result of protein of *Lysinibacillus sphaericus* against Human liver cancer cells (Hep G2) shows that at 50µg/ml, 100 µg/ml and 150 µg/ml concentrations of *Lysinibacillus sphaericus*, the percentage of viable cells was found to reduce to 28.844, 26.451 and 25.134 respectively. (Table 1,Figure 1) Also the cell viability was found to decrease in increase in concentration of protein. The anti-cancer activity of the protein was found to be nearer even at least concentration (50 µg/ml) when compared with a commercially available anti-cancer drug called Cyclophospamide at 90µg/ml of concentration has shown 24.086% of live cells when treated with the cancer cells at the same conditions.

The anti-cancer effect of protein isolated from *Lysinibacillus sphaericus* was also checked by MTT assay against Human liver cancer cells (Hep G2). The result showed 33.33%, 32.15%, 31.31% and 24% of live cells on treatment with 50µg/ml, 100µg/ml and 150 µg/ml tested protein and 90 µg/ml of Cyclophospamide. The IC_{50} values of both the tested proteins were found to be below 50µg/ml concentration.

Peptide Mass Fingerprinting (PMF) Analysis

The obtained MALDI spectrum was then searched and compared against existing databases like Swiss Prot and NCBI (National Centre for Biotechnology Information) using Mascot software (Matrix Science) and the protein is identified by comparing the molecular weight against a database of known peptides. Mascot computes the score based on the probability that the peptides from a sample match those in the selected protein database.

Results and Discussion

Isolation and Identification of Bacteria

52 different bacterial isolates were isolated by serial dilution agar plating method. All were checked for antibacterial activity against clinical pathogens. Among the 52 bacterial isolates the isolate which have shown effective growth inhibition activity against bacterial pathogens were identified as *Lysinibacillus sphaericus* by 16S rDNA sequencing **(TABLE:1)** & **(FIG:1).** The protein obtained from *Lysinibacillus sphaericus* was purified by ammonium sulphate and preparative chromatography. 40% saturated of dialyzed protein shown anti Cancer activity against HepG2 cancer cell line by MTT assay **(FIG:2)**

TABLE:1 Screening Of Antibacterial Activity Of *Lysinibacillus Sphaericus*, Supernatant and Protein from *Lysinibacillus Sphaericus*

Sl No.	Organism	Zone of inhibition (mm)		
		Active culture	Supernatant	Protein
1	*C .diphtheriae*	29	22	21
2	*K. pneumoniae*	15	-	14
3	MRSA	26	19	24
4	*Shigella* sp.	16	16	19
5	*S. aureus*	26	21	22
6	*S. epidermidis*	16	15	20

FIG:1 Screening of Antibacterial Activity of *Lysinibacillus Sphaericus*

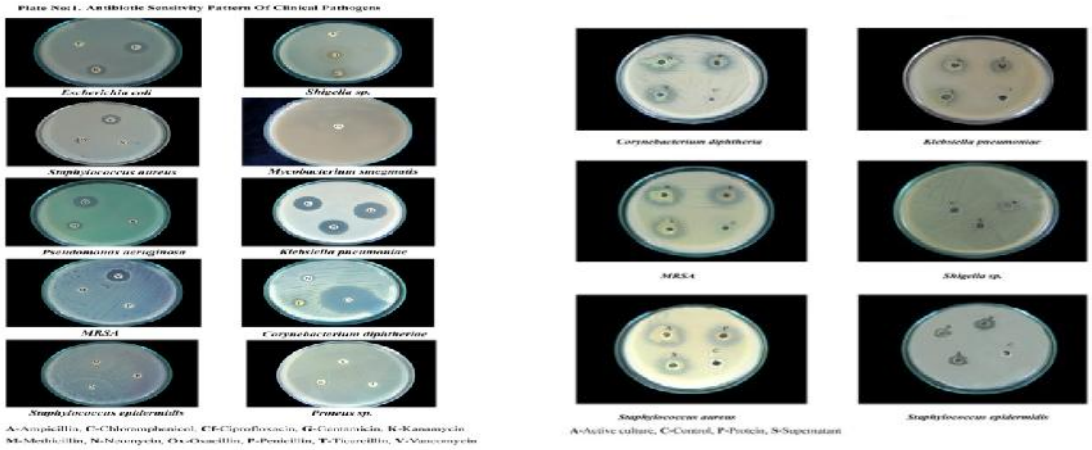
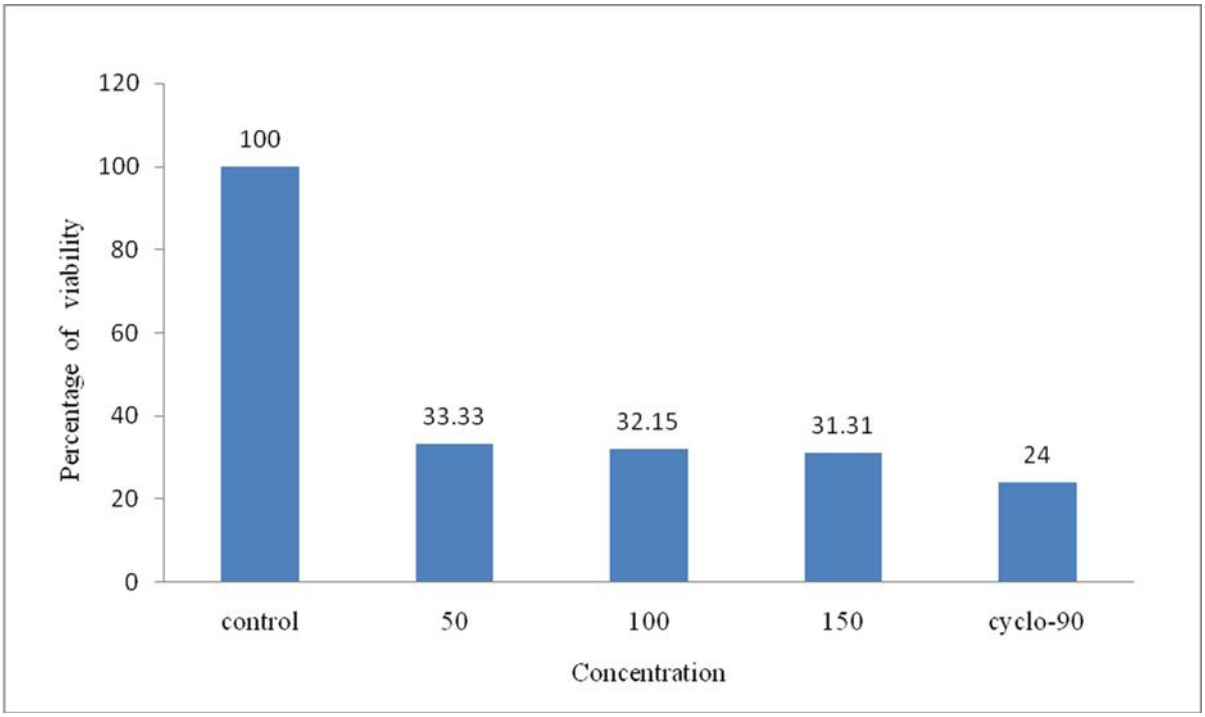


FIG:2 Percentage of Viability Shown By Hep-G2 Cancer Cell Lines on Treatment with Protein from *Lysinibacillus sphaericus*



Anticancerous Activity

Anti-cancer activity of Polybia-MP1 isolated from the venom of the social wasp Polybia paulista was determined by MTT assay against various human cancer cell lines like human bladder cancer cell lines (Biu87 & EJ), prostate cancer cell line (PC-3) and human umbilical vein endothelial cell line HUVEC. The IC_{50} values of polybia-MP1 against various cancer cell lines were found to be 64.68, 52.16, 75.51 and 55.6 for PC-3, Biu87, EJ and HUVEC cell lines respectively. [9]. The cytocidal activity of *Lysinibacillus sphaericus* protein against Hep G2 cell lines makes it possible for protein based anti cancer drugs in medical use. The direct clinical application may not be possible. The exact receptors in cancer cells can be identified and it can be targeted for the drug formulation. Thus the future work includes the screening strategies for innovative anti cancer proteins from *Lysinibacillus sphaericus*.**(TABLE:2)**

TABLE: 2 MTT Assay of Anti-cancer Protein from *Lysinibacillus sphaericus*

Conc. of Protein µg/ml	O D at 570nm	% of viability	% of Toxicity
50	0.536	28.844	33.33%
100	0.492	26.451	32.15%
150	0.467	25.134	31.31%
Cyclophosphide (90 µg/ml)	0.448	24.086	24%
Control	1.86	100	-

PMF Analysis

Mascot computes the score based on the probability that the peptides from a sample match those in the selected protein database. Protein score greater than 68 was considered significant with a value of $p<0.05$. The more peptides the Mascot identifies from a particular protein, the higher the mascot score for that protein.In Peptide Mass Fingerprinting the mass values (peptides) were subjected for homology search in protein databases. The search results showed the 5 matches among 14 query sequences. The molecular masses of matched peptides are 1168.4882, 1037.6243, 2273.0010, 2288.9946.2192 and 8508. Sequences of matched Peptides are,

M S R T V M C R K (Met- Ser-Arg-Thr-Val-Met-Cys-Arg-Lys)

L N M M N A……E D R K F L Q Q E M…… D K F L S G E D Y A……K (Leu-Asn-Met-Met-Asn-Ala……Glu-Asp-Lys-Phe-Leu-Gln-Gln-Glu-Met…..Asp-Arg-Lys-Phe-Leu-Ser-Gly-Glu-Asp-Tyr-Ala……Lys).This reveals the novel composition of the anticancer protein obtained from *Lysinibacillus sphaericus* **(FIG:3)**

FIG: 3 Mascot Search Results for PMF

Protein View
Match to: **FETP_PSEA7** Score: **47** Expect: **3.2**
Probable Fe(2+)-trafficking protein OS= *Lysinibacillus sphaericus* **(strain PA7) GN=PSPA7_5885 PE=3 SV=1**
Nominal mass (Mr): **10675**; Calculated pI value: **6.10**
NCBI BLAST search of FETP_PSEA7 against nr
Unformatted sequence string for pasting into other applications
Taxonomy: *Lysinibacillus sphaericus* PA7
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **14**
Number of mass values matched: **5**
Sequence Coverage: **40%**

Matched peptides shown in **Bold Red**
1 **MSRTVMCRK**Y HEELPGLDRP PYPGAKGEDI YNNVSRKAWD EWQKHQTMLI
51 NERR**LNMMNA EDRKFLQQEM DKFLSGEDYA K**ADGYVPPSA
Residue Number Increasing Mass Decreasing Mass
Start - End Observed Mr(expt) Mr(calc) ppm Miss Sequence
1 - 9 1168.4882 1167.4809 1167.5675 -74 2 -.MSRTVMCRK.Y
2 - 9 1037.6243 1036.6170 1036.5270 87 2 M.SRTVMCRK.Y
55 - 72 2273.0010 2271.9937 2272.0286 -15 2 R.LNMMNAEDRKFLQQEMDK.F 2 Oxidation (M)
55 - 72 2288.9946 2287.9873 2288.0235 -16 2 R.LNMMNAEDRKFLQQEMDK.F 3 Oxidation (M)
64 - 81 2192.8508 2191.8435 2192.0459 -92 2 R.KFLQQEMDKFLSGEDYAK.A Oxidation (M)
No match to: 906.4180, 995.4596, 1020.4265, 1097.9470, 1138.6760, 1153.4830, 1229.6719, 1490.6354, 2162.9126

Conclusion

In addition to Anti Cancer activity, the protein possesses the Anti MRSA activity which is a major concern in clinical laboratory. The cytocidal activity of *Lysinibacillus sphaericus* protein against Hep G2 cell lines makes it possible for protein based anti cancer drugs in medical use. The direct clinical application may not be possible. The exact receptors in cancer cells can be identified and it can be targeted for the drug formulation. Thus the future work includes the screening strategies for innovative anti cancer proteins from *Lysinibacillus sphaericus.*

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