DETECTION OF ANTIMICROBIAL EFFECT OF EXTRACELLULAR PROTEIN PRODUCED BY PSYCHROPHILIC ACTINOMYCETES ISOLATED FROM MANALI ICE POINT

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Abstract
The present study reveals that the effect of extra cellular protein isolated from Psychrophilic Actinomycetes against clinical pathogens. Totally 13 Actinomycetes were isolated from glacier soil. Among them the genus Streptomycetes and Micromonospora were isolated highly. MM9 medium was used as fermentation medium and the antimicrobial peptide activity was performed by disc diffusion method. Out of 13 isolated actinomycetes C2, I and J were found to be effectively produce antimicrobial Extracellular protein active against 3 test pathogens. The most important significant finding of this work is antimycobacterial activity of extracellular protein produced by Streptomycetes (C2) and Micromonospora sp (J) were active against Mycobacterium tuberculosis. The zone of inhibition was 20 mm (C2) and 22mm (J) respectively. Among the C2, I and J isolates Micromonospora sp (J) is most effective towards all three test organism. The molecular weight of antimycobacterial protein was 39,000 Da (C2) and 3000 Da (J).

Keywords: Anti microbial Peptides, Psychrophiles, Mycobacterium, Drug resistance, Micromonospora sp

1. Introduction
Soil microorganisms provide an excellent resource for the isolation and identification of therapeutically important products. Among them actinomycetes are important groups. Actinobacteria originally considered as an intermediate group between bacteria and fungi, but later it has attained a distinct position. Actinobacteria are a well defined group of gram positive, aerobic, free living bacteria1. Actinomycetes are prokaryotes having high G+C content in their DNA, with various metabolic possibilities. The metabolic diversity of the actinomycetes family is due to their extremely large genome, which has hundreds of transcription factors that control gene expression, allowing them to respond to specific needs5. Studies of Microbiology in glaciers initiated in the 1960s. At the initial stage all work was focused on studies on genera, quantity and distribution of microorganisms. During the last two decades more information has been obtained about the diversity and distribution and extensive study has also been done on the physiological and genetic traits and mechanism of cold adaptation of microorganisms in glaciers3. This progress has led to a declining trend in the discovery of unknown natural products derived from microorganisms4, despite identification of only an estimated 1–3% of the existing compounds produced by Streptomyces species5. Programmes aimed at the discovery of antibiotics and other bioactive metabolites from microbial sources have yielded an impressive number of compounds over the past 50 years, many of which have found applications in human medicine and agriculture. However, it soon became apparent during screening programmes that some microbial metabolites were discovered more frequently than others. As the number of described microbial metabolites increased, so did the probability of rediscovering known compounds. At the present time, with several thousands of described microbial metabolites, approach must be introduced into screening programmes to increase the chances of discovering novel compounds6. Actinomycetes are the strongest antagonists among microorganisms. The antibiotic substances they produce display antibacterial, antifungal, antitumor,
antiprotozoic, and antiviral properties. Of the ten thousand known antibiotics produced by microorganisms over a decade ago, about 70% are of actinomycete origin; of them, representatives of the genus *Streptomyces* account for two thirds\(^7\). Actinomycetes from the genus *Micromonospora* occupy the second place in this list: they produce over 300 broad-spectrum antibiotic substances\(^8\). Antimicrobial peptides are generally between 12 and 50 amino acids. These peptides include two or more positively charged residues provided by arginine, lysine or, in acidic environments, histidine, and a large proportion (generally>50%) of hydrophobic residues. The secondary structures of these molecules follow 4 themes, including i) α-helical ii) β-stranded due to the presence of 2 or more disulfide bonds, iii) beta-hairpin or loop due to the presence of a single disulfide bond and/ or cyclization of the peptide chain, and iv) extended\(^9\). Many of these peptides are unstructured in free solution, and fold into their final configuration upon partitioning into biological membranes. It contains hydrophilic amino acid residues aligned along one side and hydrophobic amino acid residues aligned along the opposite side of a helical molecule\(^10\). This amphipathicity of the antimicrobial peptides allows to partition into the membrane lipid bilayer. The ability to associate with membranes is a definitive feature of antimicrobial peptides\(^11\) although membrane permeabilisation is not necessary. These peptides have a variety of antimicrobial activities ranging *Mycobacterium tuberculosis*, the etiological agent of tuberculosis, kills more than 2 million people every year worldwide in concurrence with HIV-related infections. Moreover, appearance of multi-drug resistant (MDR) strains of *M. tuberculosis* to many, if not all, of the existing drugs has been noted. This has necessitated the development of novel anti tubercular agents\(^12\). Tuberculosis (TB) is the most common cause of death due to a single infectious agent worldwide in adults. In 1993, the World Health Organization (WHO) took an unprecedented step and declared TB to be a global emergency\(^13\). According to the recent estimates, one third of the human population (about 1.86 billion people) was infected with *Mycobacterium tuberculosis* worldwide in 1997. The organism causing TB was described only a century ago by Robert Koch on 24th March 1882. Until middle of the 20th century, there was no definitive treatment available for TB. With the availability streptomycin, isoniazid and paraaminosalicylic acid (PAS), in the mid 1940s, predictable, curative treatment for TB became a reality\(^14\). The introduction of rifampicin, pyrazinamide and ethambutol in the subsequent years ushered in the era of short-course treatment. In 1943, an American named Selman Waksman, together with his co-workers, discovered that a fungus called *Streptomyces griseus* produced an antibiotic substance which they named "streptomycin". Streptomycin was the first antibiotic used against *M. tuberculosis*\(^15\).

The global resurgence of TB and the rapid emergence of MDR-TB have motivated the research of novel anti tubercular agents and a lot of top researchers in various fields are doing their best to investigate novel compounds with anti-tubercular activity\(^16\). Actinomycetes are the main source of clinically important antibiotics, most of which are too complex to be synthesized by combinatorial chemistry, making three quarters of all known products; the *Streptomyces* are especially prolific, producing around 80% of total antibiotic products. *Micromospora sp* is the runner up with less than one-tenth as many as *Streptomyces sp*\(^17\).  

2. Materials and Methods

2.1. Sample collection: The soil sample was collected from the Ice Point of Rothang Hill, HIMACHALPREDISH the gate way of Himalayas Situated at an altitude of 4179 mtrs above from the sea level on October 2008. The samples were collected in a sterile container and brought up to the laboratory in ice bag. 

2.2. Isolation of Actinomycetes from glacier soil sample: The serial dilution set up was prepared and sterilized and the soil samples were brought up to the laboratory condition. One gram of soil sample was serially diluted up to 10\(^8\) dilution. The dilution such as 10\(^-3\), 10\(^-4\), and 10\(^-5\) were taken and plated on Actinomycetes agar medium. The plate is then incubated at 15°C for 21 days until the colonies were developed. Based on the nature of colony characters the actinomyces was selected and purified by sub cultured on Starch casein nitrate agar supplemented with cyclohexamidine (0.01mg/ml). 

2.3. Phenotypic characteristics: Isolated strains were identified through morphological, biochemical and chemotaxanomical studies as follows.
2.3.1. Morphological study: Isolates grown facultatively selected and their colony morphology was studied by allowing them to grow on Starch casein Nitrate agar and Actinomycetes agar. All the plates were prepared at one week time intervals for proper screening. The plates are streaked with the isolates and kept at 20°C for 10 days. The growth was recorded on 5th and 10th day interval. Nature of colony, color of colony, pigmentation and spore mass were recorded and tabulated.

2.3.2. Slide culture method: This technique is used to find out the spore morphology and type of mycelium produced by the isolated strains. Actinomycetes agar plates supplemented with cyclohexamide were prepared; 4x4mm of agar was sliced and placed over the surface of sterile glass slide on moist chamber with 60% ethanol. A clean cover glass sterilized by 90% ethanol was kept on each agar blocks. Isolates were inoculated on the four corners and incubated at 35°C for 2-3 days. End of the third day the plates were taken out from incubator and the cover glasses were stained by Sudan black IV. The stained cover glass air dried and mounted on new clean glass slide and was examined under Nikon photomicroscope. Aerial mycelium was observed as stained and the unstained are substrate mycelium.

2.3.3. Biochemical studies: Various Biochemical tests were performed for the identification of potent isolates are as follows; indole, methylred and vogues proskaur, citrate utilization and Hydrolysis of starch.

2.3.4. Utilization of sugar and acid production: This was performed according to the method of Lechevalier with minor modifications. The sugar utilization medium were prepared and sterilized. Six different sugar compounds (arabinose, inositol, lactose, mannitol, mannose, xylose) were taken and 1% concentration of sugar was taken and added to their labelled medium respectively. After the sugar compounds were added to the medium. The isolated Actinomycetes were inoculated in their respective medium. After inoculation the tubes were incubated at 35°C for 2 days. After incubation the tubes were observed and the results were tabulated.

2.3.5. Optimization of growth condition: Minimal medium 9 was taken for the production of antimicrobial peptide. Small disc of fresh actinomycetes isolates were inoculated separately on sterile 250 ml minimal medium. The inoculated flasks were kept under 75rpm, 125 rpm, and 150rpm at 15°C, 28°C and 35°C for 24hrs days. Similar set up was prepared and kept under 75rpm, 125 rpm, and 150rpm at. The agitation was given only from the day time from 8 am to 8 pm. After 5 days incubation the broth was transferred into sterile BOD bottle with glass beads and was shaken vigorously to break up the spore and mat like growth and the OD values were taken at 550 and 365 nm.

2.3.6. Fermentation: About 500 ml of Minimal medium were prepared and sterilized. The isolated Psychrophilic Actinomycetes were inoculated on ISP4 broth and then incubated at 35°C for 7 days under 150 rpm.

2.3.7. Isolation of extra cellular Protein from Actinomyetes Grownon MM 9 medium: Two different methods were used to isolate the protein from fermented MM 9. the broth was centrifuged at 5000rpm to remove the cells and mycelium and the supernatent was filtered through whatmann filter paper. The isolated protein pH was also noted.

a) TCA method:
Methodology: 10 volumes of 10% TCA in acetone was mixed with culture filtrate sample and kept overnight at refrigerator. 10% of cold acetone was added, vortex and stand at -20°C 10 minutes. The mixture was centrifuged at 15000rpm for 5 minutes. Remove supernatent allowed pellet to dry. Prevent complete desiccation of the protein pellet.

b) Methanol chloroform method:
Methodology: 4 volume of methanol was added and vortex well. 1 volume of chloroform was added and vortex. 3 volume of distilled water was added and vortex. This mixture was centrifuged for 2 minutes at 15000 x g the protein should be at the liquid interface, removed aqueous top layer and added 4 volume of methanol vortex. Centrifuged 2 minutes at 15000xg, remove as much liquid as possible without disturbing precipitates, speed-vac samples to dryness (or) dry under nitrogen.

2.4 Total protein estimation by Lowry’s method: 0.05-1mg/ml of standard bovine serum albumin was prepared. 0.2ml of standard solution was taken and mixed with 2ml of alkaline copper sulphate reagent and incubated for 10minutes. 0.2ml of folin reagent are added to each tube and incubated for an hour. The unknown sample was diluted and a known volume of sample was treated with alkaline copper and folin reagent. The OD values of standard and sample was taken at 650 nm and plotted on graph.
2.5 Screening of antibacterial activity of isolated Actinomycetes (Disc diffusion method): About 50 µg of isolated protein was loaded on sterile disc and allowed to air dry overnight at chamber. Dubos medium for mycobacterium sp and Muller Hinton agar plates for other two test organism was prepared and the respective test pathogens were swabed over the surface of agar plates. The protein disc was placed over the surface of test organism swabbed plates. Muller hinton agar plates were incubated at 37°C for 24 hrs and the mycobacterium plates were kept under anaerobic jar for 3 weeks. The zone of inhibition was recorded and tabulated. The antimycobacterial molecular weight was estimated in 1.8 mg/ml in Vibrio cholerae (5.1 mg/ml) and the least concentration was 3000 Da. The molecular weight of antimycobacterial protein produced by Streptomyces sp was 39,000 Da and Micromonospora sp were 3000 Da. The properties of the antimicrobial peptides show, that they are evolutionarily conserved, ubiquitous, simple and effective factors acting within the innate immune system. Moreover, because of their specificity and safety they seem to be suitable for medical use. Several peptides and their derivatives have already passed clinical trials successfully.

3. Results and discussion
A Total of 13 Actinomycetes were isolated from manali soil sample by pour plates technique and suspected colonies were aseptically maintained in Actinomycetes agar plates. All the isolates were capable to grow an SCNA, actinomycetes agar, starch agar and ISP 2. The luxuriant growth was showed only on SCNA and Actinomycetes agar. All the 13 strains are differed on their colony morphology and mass of spore color (table 1). The genera of active isolates were determined by its spore and colony morphology (Table 2). All the isolated actinomycetes showed luxurious growth under 150 rpm at 28°C (Table 3). Proper identification of genera and species of actinomycetes, besides morphological and physiological properties18, various other biochemical properties such as cell wall chemo type, whole-cell sugar pattern, peptidoglycan type, phospholipids type and G+C% of DNA should be determined. Likewise, the statement of Thirumalachar and Sukapure19 that Micropolyspora is differentiated from Streptomyces "only by the fragmenting nature of the vegetative mycelium" is hardly appropriate, either from a morphological or from a chemical point of view.

The extra cellular protein from the fermentation medium was effectively isolated by Methanol chloroform than TCA method. The total yield of crude protein also higher in methanol method (Table 5). The pH of the protein was ranges 6 to 8 (table 4). The concentration of total protein was higher in Micromonospora Sp (5.1 mg/ml) and the least concentration was estimated in1.8 mg/ml in Streptomyces Sp and micropolyspora Sp (Table 4).

The entire 13 isolated protein sample shows antibacterial activity against Amp resistant E. coli but not against Vibrio and Mycobacterium strains. Out of 13 only 3 were significantly active against this test pathogens. The Amp resistant E. coli was highly sensitive to isolate J and it was identified as Micromonospora sp. Similarly isolates C2 and J were active against Mycobacterium tuberculosis bacilli (Plate 1). The zone of inhibition was measured as 20 and 22 mm respectively to C2 and J. both the active isolates were identified as Streptomyces sp (C2), and Micromonospora sp (J). Daptomycin is a cyclic lipopeptide antibiotic produced by Streptomyces roseosporus active against Mycobacterium bovis was reported by Richard20. The other test pathogen Vibrio cholerae highly sensitive to isolate designed as 1 (Micropolyspora sp) and J. The zone of inhibition was 13 and 15 mm in diameter. Figure 1 show the isolate Micromonospora sp (J) capable to produce more amount of extra cellular protein than C2 and I. The active strains C2 and J were selected to characterize it protein nature and the active antimycobacterial protein shows single band (plate 2) which was compared with Known marker. The molecular weight of antimycobacterial protein produced by Streptomyces sp was 39,000 Da and Micromonospora sp were 3000 Da. The properties of the antimicrobial peptides show, that they are evolutionarily conserved, ubiquitous, simple and effective factors acting within the innate immune system. Moreover, because of their specificity and safety they seem to be suitable for medical use. Several peptides and their derivatives have already passed clinical trials successfully.

References
1. Pandey, B., Ghimire, P and Agrawal, V.P. International Conference on the Great Himalayas: Climate, Health, Ecology, Management and Conservation, Kathmandu, Organized by Kathmandu University and the Aquatic Ecosystem Health and Management Society, Canada from the soil of Zahedan country, South-east of Iran. Irn J Med Sci. 2004: 24, 65-67.
2. Goshi, K.T., uchida, A., Lezava, M., Yamasaki, K., Hiratsu. Telomerase and terminal inverted repeat of the linear chromosome of Streptomyces griseus. J of Bacteriol 2002:184, 3411-3415.
3. Zlatanov, M., Pavlova, K., Grigorova, D. Lipid composition of some yeast strains from
Livingston Istand, Antarctica. *Folia Microbiol* 2001: 46,402-406

4. Watve, MG. Tickoo, R., Jog, M.M and Bhole, B.D. How many antibiotics are produced by the genus *Streptomyces*? Arch. Microbiol 2001: 176, 386–390.

5. Baltz, R.H. Marcel Faber Roundtable: is our antibioticpipeline unproductive because of starvation, constipation or lack of inspiration? *J. Ind. Microbiol. Biotechnol*2006: 33, 507–513

6. Busti, E., Monciadini, P., Cavaletti, L., Bamonte, R., Lazzarini, A., Sosio, M and Donadio, S. Antibiotic-producing ability by representatives of a newly discovered lineage of Actinomycetes. *Microbiol* 2006:152, 675–683

7. Miyadoh, S . Research on antibiotic screening in Japan over the last decade: A producing microorganisms approach. *Actinomycetologica* 1993: 9: 100-106

8. Vobis, G. A handbook of the biology of bacteria: ecophysiology, isolation, identification, applications, Balows A., Truper HG., Dworkin M and Harder W. Eds., New York: Springer, 1992: (2),1029–1060.

9. Dhople, V., Kru kemeyer, A and Ramamoorthy, A. "The human beta-defensin-3, an antibacterial peptide with multiple biological functions", *Biochimica Biophysica Acta – Biomembranes* 2006: 1758 (9), 1499–1512.

10. Yeaman, M.R and Yount, N.Y. "Mechanisms of antimicrobial peptide action and resistance". *Pharmacological reviews* 2003:55(1), 27–55

11. Hancock, R.E and Chapple, D.S. Peptide antibiotics. *Antimicrob Agents Chemother* 1999: 43, 1317–23.

12. Tomioka, H and Namba, K. Development of antitubercular drugs: current status and future prospects. *J. Infect. Dis. 2007:196(1), 86-107

13. Grange, J.M. and Zumla, A.. The global emergency of tuberculosis: what is the cause? *J R Soc Health 2002: 122, 78-81

14. Sharma, S.K and Mohan, A. Multidrug-resistant tuberculosis. *Mediquest* 1995:13, 1-11.

15. Zetterstrom, R. Selman A. Waksman (1888-1973) Nobel Prize in 1952 for the discovery of streptomycin, the first antibiotic effective against tuberculosis. Acta. Paediatr 2007:96

16. Biava, M., Porretta, G.C and Poce, G.1,5-Diphenylpyrrole derivatives as antimycobacterial agents. Probing the influence on antimycobacterial activity of lipophilic substituents at the phenyl rings. *J. Med. Chem* 2008:51, 3644-3648.

17. Lam, K.S. Discovery of novel metabolites from marine actinomycetes. *Curr. Opin. Microbial* 2006:9, 245-251.

18. Kutzner, A. Erosion and the origin of charged andneutral species in vacuum arcs. *Acta microbiol* 1972: 9,245-251.

19. Thirumalachmar, J and Sukapurre, S. Studies on species of the genus *Chainia* from India. *Hindustan Antibiotics Bulletin* 1964: 6, 157-166

20. Richard, H., Baltz, Paul Brian, Vivian Miao and Stephen K.Wrigley. Combinatorial biosynthesis of lipopeptide antibiotics in streptomycyes roseosporus journal of industrial microbiology & biotechnology 2005:33(2), 66-74.

21. Levy, O. Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. *Blood* 2000: 96, 2664–72.

### Table 1: Growth of Actinomycetes on Actinomycetes Agar and Starch Casein Agar Media:

| STRAIN CODE | ACTINOMYCETES AGAR  | STARCH CASEIN AGAR |
|-------------|---------------------|---------------------|
| A1          | white powdery Colonies | Dull white powdery colonies |
| A2          | Dull White mat like growth Colonies | Pale yellow mat like colonies |
| A3          | Pale Yellow Mucoid Colonies | Pale Yellow to Yellow Mucoid Colonies |
| B           | Chalky white fine powdery Colonies | Dull white powdery colonies |
| C1          | Ash rough powdery Colonies | Ash rough powdery Colonies |
| C2          | Whitish Grey Powdery Colonies | Ash Powdery Colonies |
| D           | Dull white To White Powdery Colonies | Ash Powdery Colonies |
| E           | Grey With White Powdery Colonies | Light Ash Powdery Colonies |
| F           | Grayish white powdery Colonies | Light Ash Powdery Colonies |
| G           | Pale Yellow to white powdery Colonies | Yellowish white powdery Colonies |
| H           | milky white powdery colonies | Light Ash Powdery Colonies |
| I           | White powdery colonies With Yellow Pigmentation | Whitish greypowdery Colonies |
| J           | White Powdery Colonies | Light Ash Powdery Colonies |
Table 2: Study of spore and morphology of isolated Actinomycetes

| Strain Code | TYPE OF MYCELIUM | SPORE MORPHOLOGY                                      | POSSIBLE GENUS        |
|-------------|------------------|-------------------------------------------------------|-----------------------|
| A1          | AM/SM            | Rarely branched, septate hyphae, Monospore            | Micromonospora sp     |
| C2          | AM/SM            | Medium length chain of spores                          | Streptomyces sp       |
| D           | AM/SM            | Long chain spore                                       | Streptomyces sp       |
| H           | AM/SM            | Long chain spore                                       | Streptomyces sp       |
| I           | AM/SM            | Rarely branched, Fragmented hyphae, short chains of conidio spore. | Planomonospora sp     |
| J           | AM/SM            | Monospore, Septate hyphae                             | Micromonospora sp     |

Table 3: Growth rate of Actinomycetes on MM9 medium

| Strain Name | OD VALUE | 15°C | 28°C | 35°C |
|-------------|----------|------|------|------|
|              | 75rpm    | 150rpm | 250rpm | 75rpm | 150rpm | 250rpm | 75rpm | 150rpm | 250rpm |
| A1          | 0.03     | 0.26   | 0.38  | 0.42  | 0.48   | 0.46   | 1.38  | 1.51   | 0.78   |
| A2          | 0.02     | 0.22   | 0.32  | 0.32  | 0.36   | 0.36   | 1.32  | 1.56   | 0.62   |
| A3          | 0.01     | 0.18   | 0.31  | 0.41  | 0.44   | 0.41   | 0.41  | 1.35   | 0.51   |
| B           | 0.021    | 0.26   | 0.28  | 0.36  | 0.38   | 0.38   | 1.36  | 1.40   | 1.16   |
| C1          | 0.016    | 0.17   | 0.27  | 0.37  | 0.41   | 0.40   | 0.67  | 1.12   | 0.57   |
| C2          | 0.03     | 0.28   | 0.28  | 0.32  | 0.39   | 0.36   | 0.28  | 1.20   | 0.38   |
| D           | 0.03     | 0.13   | 0.23  | 0.33  | 0.39   | 0.35   | 0.63  | 1.81   | 0.43   |
| E           | 0.032    | 0.20   | 0.28  | 0.33  | 0.41   | 0.39   | 0.43  | 1.56   | 0.53   |
| F           | 0.040    | 0.31   | 0.36  | 0.41  | 0.42   | 0.41   | 0.51  | 1.04   | 0.51   |
| G           | 0.06     | 0.28   | 0.33  | 0.38  | 0.38   | 0.37   | 0.58  | 1.22   | 0.58   |
| H           | 0.04     | 0.14   | 0.22  | 0.34  | 0.36   | 0.38   | 0.44  | 1.15   | 0.46   |
| I           | 0.05     | 0.15   | 0.25  | 0.35  | 0.36   | 0.39   | 0.45  | 0.97   | 0.46   |
| J           | 0.08     | 0.18   | 0.21  | 0.38  | 0.38   | 0.44   | 0.48  | 1.02   | 0.48   |

Table 4: Yield of isolated protein per 2ml culture filtrate

| Strain Name | Initial weight | Final weight | Concentration of Protein (mg)/5Ml | Methanol and chloroform | TCA method | pH |
|-------------|----------------|--------------|-----------------------------------|-------------------------|------------|----|
| A1          | 1.069          | 1.147        | 78mg                              | 98mg                    | 7          |
| A2          | 1.069          | 1.129        | 60mg                              | 96mg                    | 7          |
| A3          | 1.069          | 1.125        | 56mg                              | 56mg                    | 7          |
| B           | 1.069          | 1.138        | 69mg                              | nil                     | 7          |
| C1          | 1.069          | 1.109        | 40mg                              | nil                     | 9          |
| C2          | 1.069          | 1.129        | 60mg                              | 46mg                    | 9          |
| D           | 1.069          | 1.140        | 71mg                              | 72mg                    | 7          |
| E           | 1.069          | 1.106        | 37mg                              | 65mg                    | 8          |
| F           | 1.069          | 1.173        | 104mg                             | nil                     | 7          |
| G           | 1.069          | 1.105        | 36mg                              | 32mg                    | 6          |
| H           | 1.069          | 1.116        | 47mg                              | 62mg                    | 7          |
| I           | 1.069          | 1.194        | 125mg                             | 43mg                    | 8          |
| J           | 1.069          | 1.116        | 47mg                              | 23mg                    | 9          |
Table 6: Antimicrobial peptide activity against *mycobacterium tuberculosis*, *amp*<sup>+</sup> *E. coli*, and *vibrio cholerae*

| Strain code | *Mycobacterium tuberculosis* | *Amp*<sup>+</sup> *E. coli* | *Vibrio cholerae* |
|-------------|-------------------------------|-----------------------------|------------------|
| A1          | -                             | 21mm                        | -                |
| A2          | -                             | 20mm                        | -                |
| A3          | -                             | 18mm                        | -                |
| B           | -                             | 18mm                        | -                |
| C1          | -                             | 19mm                        | -                |
| C2          | 20mm                          | 23mm                        | -                |
| D           | -                             | 21mm                        | -                |
| E           | -                             | 16mm                        | -                |
| F           | -                             | 17mm                        | -                |
| G           | -                             | 18mm                        | -                |
| H           | -                             | 21mm                        | -                |
| I           | -                             | 21mm                        | 12mm             |
| J           | 22mm                          | 27mm                        | 16mm             |

Table5: Protein Estimation by Lowry *et al* method

| Concentration of Standard | OD VALUE |
|---------------------------|----------|
| 0.25                      | 0.06     |
| 0.5                       | 0.145    |
| 1                         | 0.311    |
| 2                         | 0.465    |
| 3                         | 0.522    |
| 4                         | 0.783    |
| 5                         | 0.954    |
| Sample C2                 | 0.432    |
| Sample I                  | 0.428    |
| Sample J                  | 0.52     |

Figure 1: Estimation of Total protein
PLATE 1 Antimycobacterial activity of isolated extra cellular protein on Dubos Agar plate

PLATE 2: Characterization active antimycobacterial protein on SDS PAGE