Purification and characterization of the antibiotic metabolite and testing for antimicrobial potential

1Tirupathi Rao A, 2Srinivas N, 3Veerendra Kumar B
1Research Scholar, 2Scientist, 3Professor
Department of Biotechnology, GITAM Institute of Science, GITAM University, Visakhapatnam, Andhra Pradesh, India

***Abstract*- Bacteria isolated from marine sediment has been evaluated for its bioactivity which showed broad spectrum antimicrobial activity against pathogenic microorganisms. Bioactive compound producing microbes has been identified by agar diffusion method. Secondary metabolite production of the microbe, the bioactive compound has been separated through chromatographic techniques. Column chromatography was used to isolate the bioactive compound, which was characterized to be by various spectroscopic techniques such NMR, IR and LCMS and it was found to be apparently bioactive compound 2-[2-Amino-5-(formyl-hydroxy-amino)-pentanoylamino]-5-({2-[2-amino-5-(formyl-hydroxy amino)-pentanoylamino]-3-hydroxy-butyryl}-hydroxy-amino) pentanoic acid. Minimum Inhibitory Concentration (MIC) of the active compound ranged from 0.5 - 15 g/mL which was found to be comparable with the standard antibiotics. Viable cell count studies of the active compound showed it to be bactericidal in nature. Further, the compound tested for its biosafety, it was found neither to be cytotoxic nor mutagenic. Cytotoxicity studies of the compound on cancer cell lines showed a valuable cytotoxic potential against all tested cancer cell lines. The current study demonstrated that the bioactive compound isolated from Staphylococcus sp. seems to be a stable and potent antimicrobial.**

Keywords **- Purification, Staphylococcus epidermidis, Chromatographic analysis, Antibiotics, Biochemical Techniques**

I. INTRODUCTION

Marine microorganisms play a significant role for the production of bioactive secondary metabolite that show various degrees of bioactivity like antimicrobial, anti-proliferative, antioxidant, bio-catalytic activities etc. The microbes have the capacity to produce multiple bioactive compounds. *Bacillus subtillis* and *Streptomyces griseus* each produce more than fifty different antibiotics. Similarly, *Staphylocccus* species also produce wide variety of bioactive compounds [1]. Chemical structure and activity of these secondary metabolites infer a wide range of possibilities i.e. antibiotics, ergot alkaloids, naphthalenes, nucleosides, peptides, phenazines, quinolines, terpenoids and some complex growth factors.

Recent techniques development makes us to further develop a high throughput bacterium that are capable of bioactive metabolite(s) production. Bacterial inoculants used for this purpose were Staphylococcus species types and were obtained from marine samples [2]. Vide literature available till date that the bioactive microbes producing N-containing hetero cycles like phenazines [3], pyrrole ring containing compounds, pyo-compounds and indole [4-6].

Mile stone for the production of pure antibiotic is extraction and purification of an extracellular bioactive compounds produced into the broth. Produced bioactive metabolite has been separated by suitable and advanced biochemical separation techniques, which is based on their specific physiochemical and biological properties. Usually, the bioactive metabolites extracted using polar and non-polar solvents, most bioactive compounds show high degree of solubility in non-polar organic solvents. Hence, the desired compound produced by the isolated microorganism has been possible using advance techniques like filtration or centrifugation, extraction of the extra cellular product by liquid-liquid extraction and high resolution techniques such as chromatography followed by concentration [7]. Literature survey decipher that there are very few reports available for the bioactive compounds produced by *Staphylococcus* species. Hence, the present study focused to purify bioactive antibiotic as produced by *Staphylococcus epidermidis* GB-51 bacterial strain and its characterization.

II. MATERIAL AND METHODS

Fermentation process for metabolite production

First step in fermentation process was the preparation of inoculum by growing Staphylococcus epidermidis GB-51 in 100 mL of nutrient broth in 250 mL conical flask in orbital shaker at 28 ^{O}C, 120 rpm for 24 h. Then 10 litres of optimized nutrient broth have been prepared and dispensed into 10 fermentation flasks of volume 5 litres each. Fill the flasks up to 1/5th volume of the conical flask's total volume with the fermentation media. Subsequently, all the media containing conical flasks were sterilized at 121 OC, 15 lbs pressure for 20 minutes. Then, cool all the media to room temperature and inoculate all the flasks with 10 mL of 18-24 h old *Staphylococcus epidermidis* GB-51 at concentration 105 CFU/mL. The fermentation process was completed at 120 rpm for 48 h on orbital shaker and conducted at fixed parameters i.e. temperature at 28 ^{O}C, pH at 7.0, dissolved oxygen (DO) at 40%. The turbidity was measured and recorded on hourly intervals by spectrophotometer. The cell free filtrate obtained after the process of centrifuging the culture at 10,000 rpm for 15 min. These filtrates were used for further process for determining the antimicrobial activity of the compound [8].

Solvent extraction for crude metabolite isolation

Crude bioactive metabolites produced in the fermentation medium has been extracted using solvent extraction method with the usage of solvents viz. acetone, chloroform, ethyl acetate, hexane, petroleum ether [9]. Vigorous mixing of the solvent with the crude ferment in the separating funnel, the resultant emulsion has been broken by passing through 50µ (microns) filter. Then aqueous and organic phases were separated using separating funnel and they were tested for their antimicrobial activity using agar well diffusion method [10]. Ethyl acetate solvent extract was selected for further studies based on its antimicrobial activity of crude. Then the identified effective crude metabolites present in active ethyl acetate phase were carefully separated from the aqueous phase and it was evaporated at 90^{O}C using Soxhlet apparatus. The crude extract obtained was concentrated by evaporation under reduced pressure at 45^{O}C in a rotary thin film evaporator. After concentration, the obtained residue was subjected to chromatographic analysis to get high pure bioactive metabolite.

Purification of crude extract by column chromatography

Column chromatography has been used to concentrate the pure bioactive metabolite. Separation has been done in 25 × 2.5 cm silica gel column matrix (100-200 µm mesh size). The column was prepared with 30 g of silica gel in a 40 cm long Corning glass tube having an internal diameter of 2.5 cm with glass wool and glass stopper at the bottom. The concentrated crude residue was dissolved in 25 mL of ethyl acetate and loaded into column. The column has been washed exhaustively with the two liters of ethyl acetate to remove similar colored impurities. Then the mixture of ethyl acetate and hexane at a ratio of 9:1, 8:2, 7:3, 6:4, 5:5 was used as gradient to elute as various fractions. The target fraction has been selected as indicated in yellow color. The active fraction was subjected to evaporation and the residue was dissolved in 15 mL of ethyl acetate to which 2 mL of absolute ethanol was added. The solution mixture was kept at 10 ^{O}C for overnight for the formation of crystals. For further crystallization the compound was dissolved in minimum quantity of ethyl acetate and then hexane was added until faint turbidity developed.

Determination of homogeneity of the antibiotic metabolite

Homogeneity of the obtained pure antibiotic was determined by one dimensional ascending thin layer chromatography (TLC) technique. It was performed on silica gel plate (TLC silica gel. 60, 20 x 20, 1.0 mm, Merck and Co., Germany) with various solvent systems. Twenty micro litres of the antibiotic solution (5 mg/mL ethyl acetate) was applied with micropipette. After solvent front reaching the 16 cm, the chromatogram was taken out from each solvent system. Then the chromatogram was visualized directly or by using U.V. light. Then the effectively eluted lanes were scraped into micro centrifuge tubes and extracted with 100% acetone. After centrifugation, the supernatants were collected to confirm their antimicrobial efficacy. Alternative method for determination of homogeneity was the combination of thin layer chromatography and overlay bio-autography (immersion bio-autography) [11] or direct autobiography. In the present investigation direct autobiography was followed [12]. For this purpose, thin layer chromatograms were developed as described above. The developed TLC plates were dipped in suspension of actively growing pathogenic bacterial cultures in proper nutrient broth and then incubated at 37^{O}C under 100% relative humid atmosphere [13]. After overnight incubation, the plates were sprayed with violet coloured tetrazolium salt (Suvidhinath Laboratories, India) solution (2 mg/mL) and incubated for 30 min; the colour change was monitored to detect growth and inhibition of the test organisms. Antimicrobial activity of the compounds was identified by the clear zones formed against a purple background on the plates [14]. The identified resulting homogenous antibiotic secondary metabolite was designated as SEGB-51.

Characterization of SEGB-51

Characterization includes physical, chemical and biological properties of the compound. State and colour were noted with naked eye. The solubility of SEGB-51 was tested with various polar and non-polar solvents. The melting point of the compound was determined by Fisher-Johns melting point apparatus.

Spectroscopy Studies

The absorption spectrum of the pure compound in acetone was determined by using UV-visible spectrophotometer (Systronics, India) to know its λmax value. The range of wave length used was 200-600 nm. The mass spectrometric analysis of SEGB-51 was performed on Finnigan MAT 95 or Varian MAT 311A Electron Ionization Mass Spectrometer. The mass range used was 0-560 m/z. The nuclear magnetic resonance analysis of compound was acquired using NMR spectrometer (400 MHz; Bruker, Germany). 1H NMR spectrum values were recorded at 400.13 MHz and 13C NMR values were recorded at 100.6 MHz. Both NMR spectra used deuterated methanol (CD_3OD) as solvent.

Determination of antimicrobial activity

Obtained pure secondary metabolite SEGB-51 was examined for antimicrobial activity to distinguish the potentiality before and after purification. Concentration of the metabolite was 1 mg/mL in acetone. Agar well diffusion method was used to determine the antimicrobial activity as described in chapter-2. The method was followed throughout the investigation which was also used for antibiotic assay.

For antibiotic assay, the purified antibiotic metabolite SEGB-51 was taken in series of dilutions (50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL and 250 µg/mL). First of all the nutrient agar plates were prepared by using agar media which were pre-inoculated with the test pathogens i.e. Gram negative Proteus vulgaris MTCC1771 and Gram positive Bacillus subtilis MTCC441 strains, obtained from MTCC, Chandigarh. The final distribution of test pathogenic cells in media was 5×106 CFU/L. By using sterile cork borer total six wells of 1cm diameter and 15mm depth were made on each agar plate. Out of 6 wells, five wells were loaded with 50 µL of each concentration and the remaining well will be the reference. The diameter of inhibition zone for each concentration was determined after the overnight incubation at 37 OC and diameter was measured by using ruler. The minimal inhibitory concentrations (MIC) were also determined for each of the test pathogens with the pure metabolite.

Statistical Analysis

The values obtained in the present investigation were analyzed and the results given as mean ± SD (Standard Deviation).

III. RESULTS

Production and extraction of crude antibiotic

Staphylococcus epidermidis GB-51 produced a pale yellow colored secondary metabolite into the production media. The produced compound was extracted comparatively more into ethyl acetate than other solvents tested viz. chloroform, diethyl ether, benzene, butanol and petroleum ether. Then the bioactive ethyl acetate extracts were dried under reduced pressure at 45 OC to obtain crude secondary metabolite in dry crystalline state. Total 3.85 g of crystals were obtained from 10 litres of pooled ethyl acetate extracts.

Purification of ethyl acetate fraction by column chromatography

The obtained crude metabolite crystals were dissolved in ethyl acetate and loaded on to column. The number of fractions eluted per solvent gradient was given in the below Table-1. The complete column run eluted total 182 fractions of each 5 mL. Based on the Rf values on thin layer chromatogram, the fractions of similar Rf value were pooled. The pooled fractions were tested for their antimicrobial efficacy by agar well diffusion method and MIC. The fraction eluted at 3:2 was shown 93% inhibition effect on microbial growth at 1% concentration. The next effective fraction was eluted at 4:1 and shown 70% inhibition at 1% concentration. So the fraction eluted at 3:2 was designated as SEGB-51 and selected for further characterization.

Table 1: Elution of ethyl acetate fraction on silica gel column

S. No.	Eluant (Ethyl acetate : hexane)	No. of fractions collected
1.	9:1	1-38
2.	4:1	39-85
3.	7:3	86-105
4.	3:2	106-158
5.	1:1	159-173
6.	Hexane	174-182

Homogeneity of the antibiotic metabolite

Thin layer chromatogram methods have been used to check the identity and homogeneity of the SEGB-51. The Rf values of the antibiotic activity possessing spots were determined and the variation in the purity of the compound and the experimental conditions used often lead to variation in the Rf values of the compound. It was observed that the purified compound formed single active spot on all chromatograms. Results of thin layer chromatography using number of solvent systems have confirmed the homogeneous nature of SEGB-51.

Table 2: Thin layer chromatographic run of metabolite from *Staphylococcus epidermidis* GB-51

S. No.	Solvent system	R_f Value
1.	Ethyl acetate (100%)	0.256
2.	Ethyl acetate-CH_2Cl_2 (9:1)	0.446
3.	Ethyl acetate-acetone (4:1)	0.645
4.	Ethyl acetate-methanol (9:1)	0.54
5.	Chloroform-acetone (4:1)	0.673

Properties and structural elucidation of SEGB-51

SEGB-51 was appeared as crystalline form with yellow colour and was soluble in ethyl acetate, ethanol, acetone, benzene and chloroform. It was almost insoluble in water hence the compound was non polar in nature. The melting point was 170 ^{O}C.

U.V. absorption spectrum

U.V. absorption spectrum of the antibiotic secondary metabolite solution showed maximum absorption at 272 nm. It may be due to the presence of aromatic ring.

Mass spectrum

The mass spectrum of SEGB-51 showed peaks at 250.17, 271.17, 289.17, 307.08, 408.2 and 548.33 m/z (Figure-14). Among the obtained peaks, the strong peak observed at 408.2 m/z was considered as base peak and infers the stable molecular configuration of compound. Elemental analysis of the compound predicted percentage of components as 26.92% carbon, 50.0% hydrogen, 8.97% nitrogen and 14.10% oxygen. Negative and positive modes of EIMS reports also suggested the molecular weight as 548.33 m/z and hence the predicted molecular formula of SEGB-51 was $C_{21}H_{39}N_7O_{11}$.

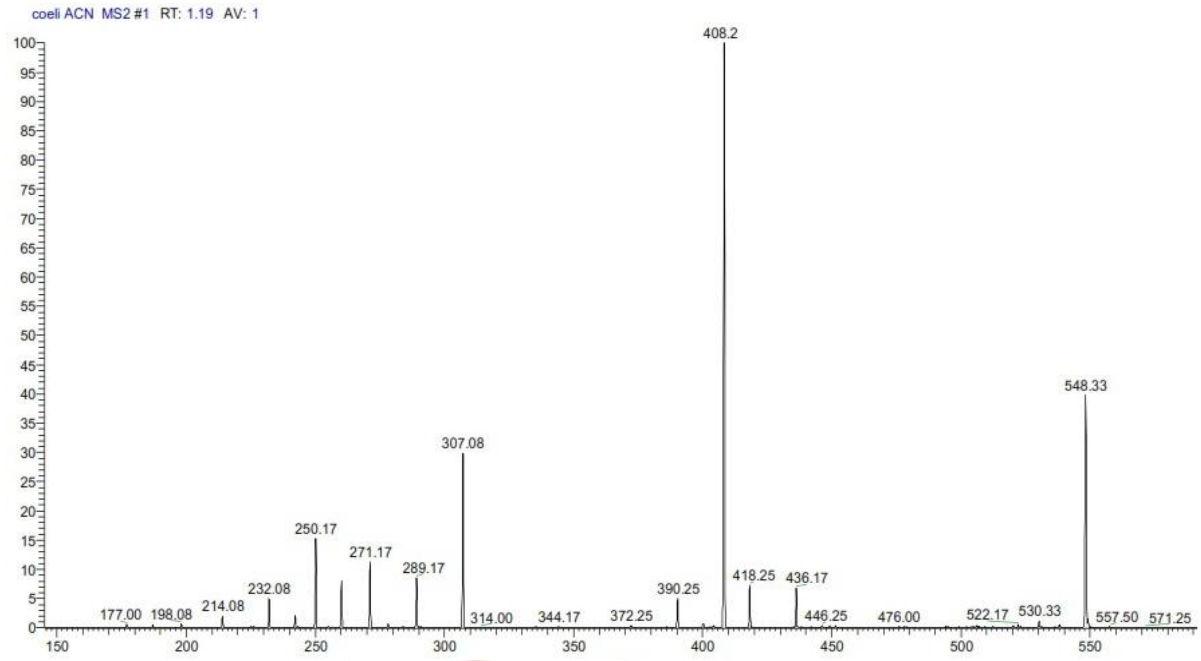

Figure 1: Mass spectrum of compound SEGB-51

NMR analysis

The NMR spectrum obtained at 400.13 MHz were presented in Figure-2 respectively. After the elucidation of the both NMR resonance analysis, the structure has been elucidated as shown in figure-3.

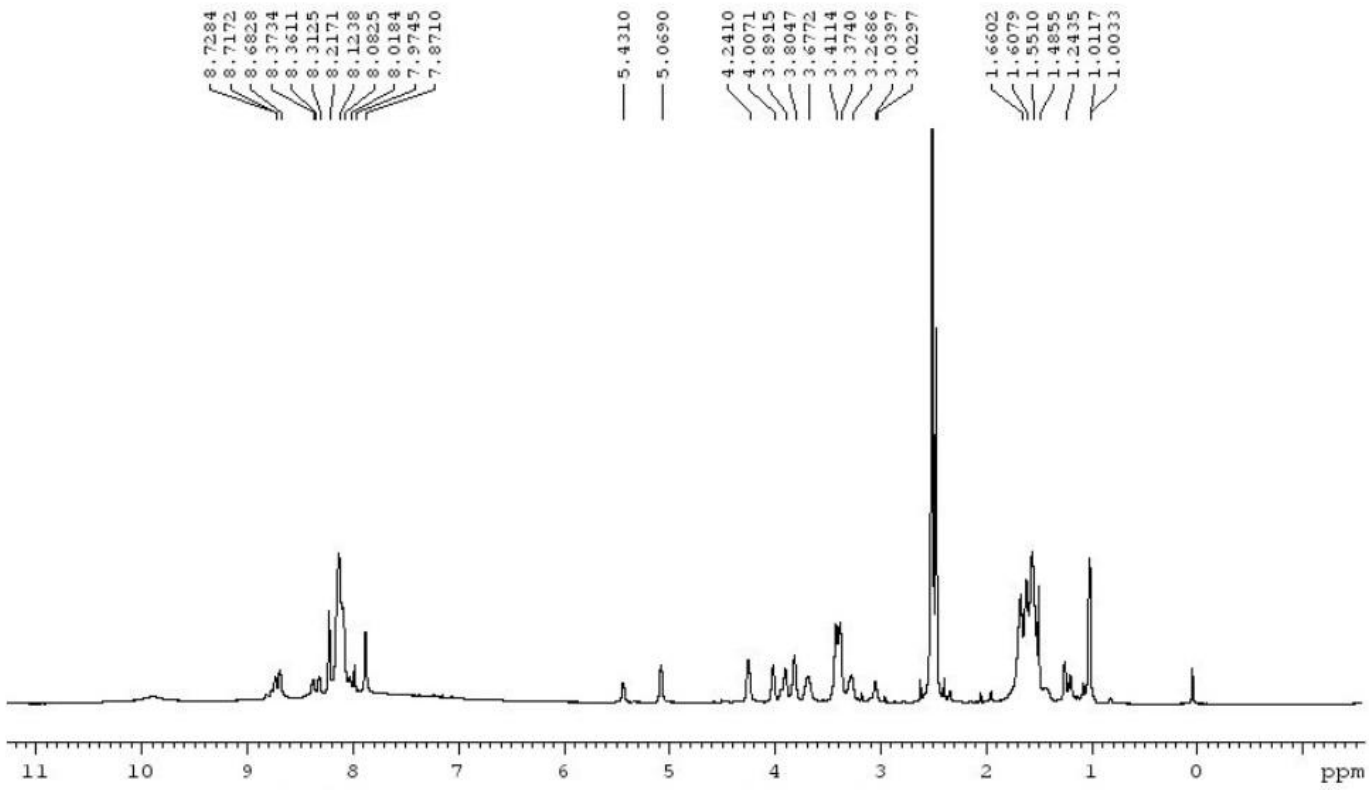

Figure-2: 1H NMR spectrum of SEGB-51 and inset structure showing position of protons

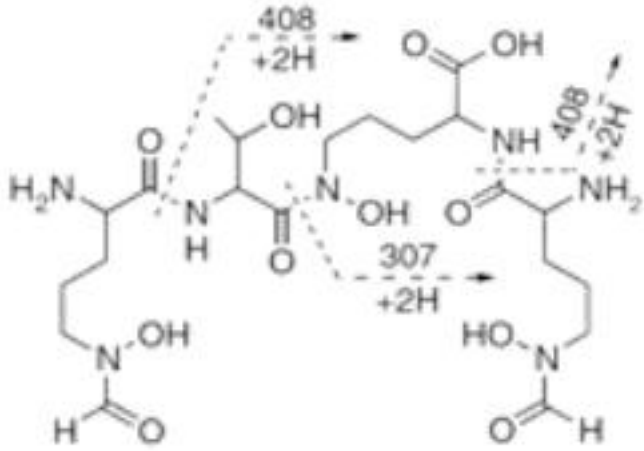

Figure 3: Structure of antibiotic SEGB-51 (2-[2-Amino-5-(formyl-hydroxy-amino)-pentanoylamino]-5-({2-[2-amino-5-(formyl-hydroxy amino)-pentanoylamino]-3-hydroxy-butyryl}-hydroxy-amino) pentanoic acid).

Results of antibiotic assay and minimum inhibitory concentration

Assay of antibiotic SEGB-51 was proved the standard antagonistic potential of it. The plot established a linear relationship between the zone inhibition diameter and concentration. As the concentration of antibiotic increased diameter of inhibition zone increased proportionately. MIC values of SEGB-51 also evident for the strength of secondary metabolite as antibiotic. It inhibited both gram positive and gram negative bacteria at low concentrations. Some of the pathogens were highly sensitive to the antibiotic SEGB-51 while some were more tolerant. The purified antibiotic SEGB-51 also showed antifungal activity. The MIC values for the bacteria and fungi were shown in the Table-24. Overall results indicated that the purified antibiotic SEGB-51 showing a broad antimicrobial activity against a wide range of pathogenic bacteria and fungi that were tested.

Table 3: Minimum Inhibitory Concentration (MIC) values of the antibiotic metabolite PFMSB-6

Test organism	**MIC (µg/mL)**
Gram -ve Bacteria	
*Pseudomonas aeruginosa (*MTCC424)	128±0.456
*Proteus vulgaris (*MTCC1771)	20±0.255
*E. coli (*MTCC443)	24±0.324
Aeromonas hydrophila	64±0.266
Aeromonas veronii	24±0.447
Pseudomonas putida	42±0.254
Pseudomonas luteola	64±0.745
Vibrio fischeri	50±0.546
E. coli	128±0.675
Gram +ve Bacteria	
Micrococcus luteus	42±0.646
*Bacillus cereus (*MTCC 430)	128±0.565
Bacillus subtilis (MTCC 441)	24±0.346
Staphylococcus aureus (MTCC 3160)	54±0.235
Fungal strains	
Candida albicans	128±0.224
Candida tropicans	0

Values expressed were a mean of the three replicates ± SD

Table-4: 13C NMR and 1H NMR spectra of SEGB-51 measured in MeOD

Carbon No.	**Chemical shift (δ)**	**Proton shift**
C 1	162.32	-
C 2	173.36	-
C 3	158.55	8.728 (1H)
C 3a	158.34	8.717 (1H)
C 3b	157.61	8.682 (1H)
C 4	29.49	8.373 (1H)

C 5	169.66	-
C 5a	168.89	-
C 6	29.28	8.688 (1H)
C 6a	29.18	8.710 (1H)
C 7	29.16	8.839 (1H)
C 8	162.32	1.902 (1H)
C 9	158.55	1.779(1H)
C 10	35.57	8.123 (2H)
C 11	55.43	1.166 (1H)
C 11a	52.17	0.608 (1H)
C 12	49.01	4.547 (3H)
C 13	70.25	2.918
C 14	65.93	4.306 (3H)
C 15	29.55	-
C 15a	27.06	1.817
C 16	35.57	1.555
C 17	52.01	0.887

IV. DISCUSSION

Staphylococcus epidermidis GB-51 was proved as the source for antimicrobial compound SEGB-51. The crude antibiotic compound showed considerable antagonistic activity against bacteria and fungi. Purification and characterization of the crude was carried out to diagnose its medicinal value. The resulted pure antibiotic showed broad spectrum of antimicrobial activity against tested bacterial and fungal pathogens.

Purification was started with solvent extraction of the crude antibiotic from the production media by using ethyl acetate as solvent. Similar method was followed for the extraction of antimicrobial compound from *Staphylococcus species* [15-16]. After drying, the compound was loaded into a silica gel column to get a pure active fraction. Purification of compounds isolated from microbes, plants etc. by column chromatography was a successful method for so many years.

Spectral analysis of the compound SEGB-51 was carried and identified it as amine derivative. U.V. spectrum elucidated the absorbance characteristics of the compound SEGB-51. Mass spectrum was elucidated the molecular weight of the compound and the molecular formula was described by elemental analysis. NMR spectral analysis confirmed the aromaticity, cyclic nature and the position of carbons present in the antibiotic compound. Similar reports were given by many researchers earlier. Based on U.V. spectral data, the polyphenolic compounds were classified as anthocyanins at 500-520 nm, hydroxycinnamic acid derivatives at 300-320 nm, flavanols have λmax at 280 nm etc. [17-19].

Current due course of study revealed that the purified compound SEGB-51 isolated from *Staphylococcus epidermidis* GB-51 strain has shown increased antagonistic activity which was confirmed by minimum inhibitory concentration values

V. CONCLUSION

The structure of the isolated compound from Staphylococcus epidermidis GB-51 was elucidated after the analysis of the available data from the EIMS, 1H & 13C NMR data. The proposed structure of the isolated pure compound named 2-[2-Amino-5-(formyl-hydroxy-amino)-pentanoylamino]-5-({2-[2-amino-5-(formyl-hydroxy amino)-pentanoylamino] -3- hydroxy-butyryl}hydroxy -amino) pentanoic acid (SEGB-51). The literature survey indicated that there were no reports on the production of SEGB-51 from microorganisms. It was a new addition to the family of alkaloid derivative antibiotics.

VI. ACKNOWLEDGMENT

The authors are grateful to GITAM University management for providing facilities required to carry out this work.

REFERENCES

[1] A. E. Namvar, B. N. A. Sara, S. Ghazaleh, S. Ghehi, P.A. Farhad bakhtiarian, H. Mahsa, Z. B. Sholeh, J. Zahra, and G. C. Sara, "Clinical characteristics of Staphylococcus epidermidis: a systematic review," GMS Hygiene and Infection Control, 2014, vol. 9(3).

[2] K. Prasanna Lakshmi, and G.M Narasimha Rao, "Some Numerical studies on Marine algae of Visakhapatnam Coast," J. Algal Biomass Utln, 2009, vol. 1(1), pp. 60 – 85.

[3] M. Widerström, "Significance of Staphylococcus epidermidis in health care-associated infections, from contaminant to clinically relevant pathogen: this is a wake-up call," J Clin Microbiol, 2016, vol. 54, pp. 1679 –1681.

[4] J. Van Eldere, W. E. Peetermans, M. Struelens, A. Deplano, and H. Bobbaers, "Polyclonal staphylococcal endocarditis caused by genetic variability," Clin Infect Dis, 2000, vol. 31, pp. 24–30.

[5] A. Nilsdotter-Augustinsson, A. Koskela, L. Ohman, and B. Soderquist, "Characterization of coagulase-negative staphylococci isolated from patients with infected hip prostheses: use of phenotypic and genotypic analyses, including tests for the presence of the ica operon," Eur J Clin Microbiol Infect Dis, 2007, vol. 26, pp. 255–265.

[6] L. G. Harris, S. Murray, B. Pascoe, J. Bray, G. Meric, L. Magerios, T. S. Wilkinson, R. Jeeves, H. Rohde, S. Schwarz, H. Lencastre, M. Miragaia, J. Rolo, R. Bowden, K. A. Jolley, M. C. Maiden, D. Mack, and S. K. Sheppard, "Biofilm morphotypes and population structure among Staphylococcus epidermidis from commensal and clinical samples," PLoS One, 2016, vol. 11, pp. e0151240.

[7] S. Linda, Thomashow, M. David, Weller, F. Robert Bonsall, and Leland S. Pierson III, "Production of the Antibiotic Phenazine-1-Carboxylic Acid by Fluorescent Pseudomonas Species in the Rhizosphere of Wheat," Applied and Environmental Microbiology, Apr. 1990, pp. 908-912.

[8] Y. L. Gordeeva, A. G. Borodkin, and E. L. Gordeeva, "Steady States of a Continuous Fermentation Process for Lactic Acid Production: The Multiplicity for a Given Dilution Rate," Theor Found Chem Eng, 2020, vol. 54, pp.482–488.

[9] A. R. Stephan, and R. M. Burgess, "Effects of sample preparation on the measurement of organic carbon, hydrogen, nitrogen, sulfur, and oxygen concentrations in marine sediments," Chemosphere, 2002, vol. 48(1), pp.139-147.

[10] Samanta, A. Samir, Dipan, Karmakar, Sanmoy, Datta, Arnab, A. Roy, K. T. Manisenthil, Roy, Dilip, Vedasiromoni, Joseph, and Tuhinadri, "Pharmacological and Biochemical studies on Telescopium telescopium - a marine mollusk from the Mangrove regions," Oriental Pharmacy and Experimental Medicine, 2008, vol. 8, pp. 386-394.

[11] Patil, G. Pawan. V. John, S. C. Charles, H. M. Gulam, F. Pierre, and V. Tibor, "Initial Measures of the Economic Activity Linked to Bangladesh's Ocean Space, and Implications for the Country's Blue Economy Policy Objectives," Journal of Ocean and Coastal Economics, 2019, Vol. 6(2), p. 2.

[12] Choma Irena, and I.M. Choma, "The Use of Thin-Layer Chromatography with Direct Bioautography for Antimicrobial Analysis," LC-GC Europe, 2005, vol. 18(9), pp. 482-488.

[13] P. Masoko, and J. N. Eloff, "Antifungal activities of six South African Terminalia species (Combretaceae)," J Ethnopharmacol, 2005, vol. 99(2), pp. 301-308.

[14] M. M. Suleimana, L. J. McGaw, V. Naidoo, and J. N. Eloff, "Detection of antimicrobial compounds by bioautography of different extracts of leaves of selected South African tree species," Afr J Tradit Complement Altern Med., 2009, vol. 7(1), pp. 64-78.

[15] T. E. Frederick, B. D. Wilson, J. Cha, S. Mobashery, and J. W. Peng, "Revealing cell-surface intramolecular interactions in the BlaR1 protein of methicillin-resistant Staphylococcus aureus by NMR spectroscopy," Biochemistry, 2014, vol. 53(1), pp. 10-12.

[16] B. Uzair, F. Menaa, B. Khan, F. Mohammad, V. Ahmad, R. Djeribi, and B. Menaa, "Isolation, purification, structural elucidation and antimicrobial activities of kocumarin, a novel antibiotic isolated from actinobacterium Kocuria marina CMG S 2 associated with the brown seaweed Pelvetia canaliculata," Microbiological Research, 2017, pp. 206.

[17] K. Aaby, R. E. Wrolstad, D. Ekeberg, and G. Skrede, "Polyphenol composition and antioxidant activity in strawberry purees; impact of achene level and storage," J Agric Food Chem, 2007, vol. 55(13), pp. 5156-5166.

[18] P. J. Gwynne, and M. P. Gallagher, "Light as a Broad-Spectrum Antimicrobial," Front Microbiol., 2018, vol. 9, pp.119.

[19] J. Medeiros-Silva, S. Jekhmane, A. L. Paioni, "High-resolution NMR studies of antibiotics in cellular membranes," Nat Commun, 2018, vol. 9(1), pp. 3963.