CHAPTER IV

RESULT AND DISCUSSION

Chapter 4

4.0 Isolation of Endophytic bacteria: A total of seven Endophytic bacteria were screened (EFB-01- EFB-07) for the production of antimicrobial metabolites from the root of *Adhatoda beddomei*. The root of this plant was chosen for research, out of all these isolates, EFB-03 strain produces maximum bioactive metabolites, in comparing to other isolates, against plant/human pathogenic fungi and bacteria. Hence, only EFB-03 has been carried forward for further research. This consistent with findings of earlier research work indicating that endophytic microorganisms can be found in virtually all higher plants (Strobel and Daisy 2003). And the endophytic colonization varies with different organs of the plant. (Schalge and Boyle 2005). Isolated endophytic bacteria were sub-cultured and preserved in the NAM medium for further Identification. The frequency of colonization (FC %) was calculated taking into account the number of morphologically distinct colonies isolated (Ni) and the total number of sample fragments inoculated (Nf) (Araujo et al. 2002).

$$FC\% = (Ni / Nf) X100$$

Bioactive metabolites of various isolated strains (EFB-01, EFB-02, EFB-03, EFB-04, EFB-05, EFB-06, and EFB-07) were tested for antibacterial activity against at least one microorganism. Bioactive metabolites of EFB-01, EFB-04, EFB-05, and EFB-07 strain, were showing very little or no zone of inhibition against different bacteria. The results are listed in Table 4.1 and bioactive metabolites of EFB-02, 03 and EFB-06 were showing high anti-bacterial properties against various bacteria. As shown in Table 4.1 and Figure 4.1.

Isolates	**Zone of inhibition against different bacteria**					
	B. subtilis	*P. aeruginosa*	*E. coli*	*S. aureus*	*R. solanacearum*	*B. cereus*
EFB-01	00	00	00	00	00	00
EFB-02	13±0.3	4±0.2	07±0.6	00	4.7±0.0	2.01±0.2
EFB-03	21±0.4	5.31±0.2	16.4±0.2	3.41±0.9	2.0±0.3	4.0±0.7
EFB-04	0.7±0.3	0.1±0.6	0.5±0.2	00	00	00
EFB-05	00	00	00	00	00	00
EFB-06	7.0±0.5	3.±0.3	6.0±0.20	00	00	0.3±0.13
EFB-07	00	00	00	00	00	00

Table 4.1: Zone of inhibition (mm) of bioactive metabolites (aqueous crude extracts) of 07 endophytic bacteria against different bacteria.

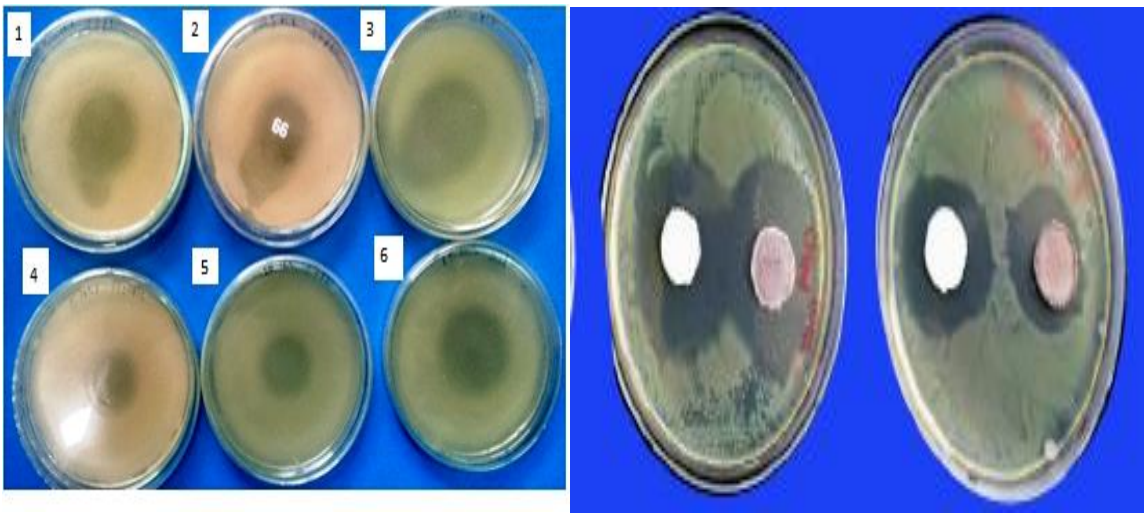

Figure 4.1: Anti- property of bioactive metabolites of endophyte bacteria (EFB-02) against different bacteria

And when using Chloramphenicol as a control, the bioactive metabolites of EFB-03 were checked anti properties against various bacteria, then EFB-03 was showing high anti-properties. The result of which is shown in Table 4.1 and Figure 4.2.

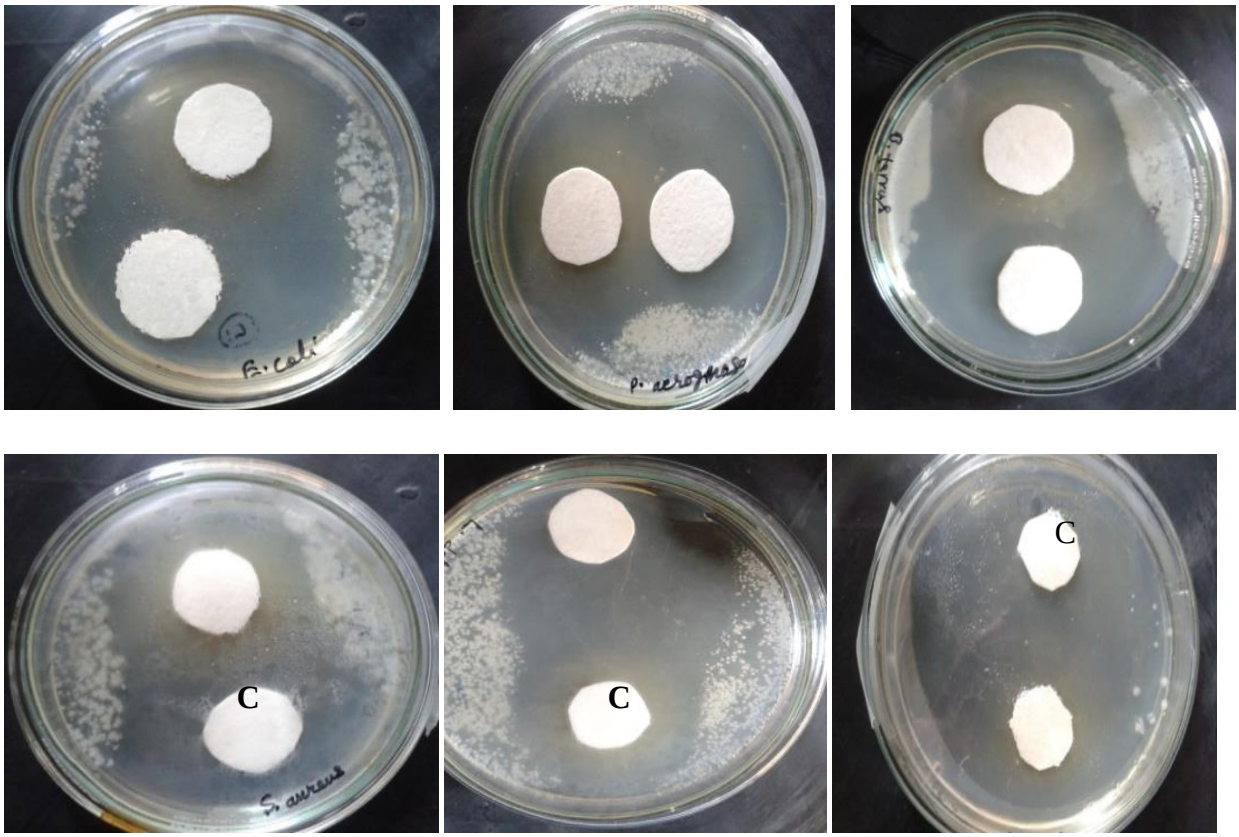

Figure 4.2: Anti-property of bioactive metabolites of endophytes bacteria (EFB-03) against different bacteria with control.

If the same process repeats for EFB-06, the bioactive metabolites of EFB-06 show a strong anti-property against various bacteria. The result of which is shown in Table 4.1 and Figure 4.3.

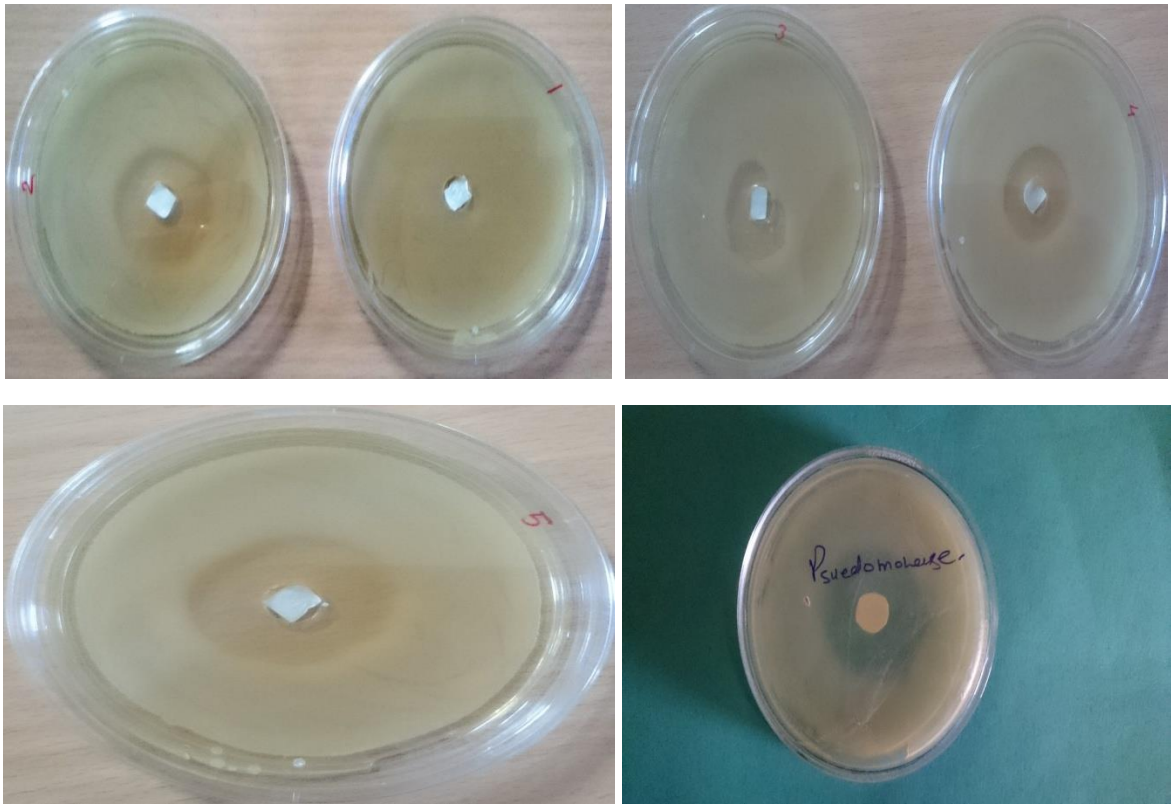

Figure 4.3: Anti-property of bioactive metabolites of endophytes bacteria (EFB-06) against different bacteria.

Similarly, the bioactive metabolites of EFB-2, EFB-03, and EFB-06 (endophytic bacteria) had positive results when tested anti-property against different fungus. EFB-1, EFB-4, EFB-05, and EFB-07 showed no antifungal activity against individual fungi and the results are shown in Table 4.2.

Isolates	zone of inhibition against different fungus		
	C. albicans	***P. chrysogenum***	***P. exigua***
EFB-01	00	00	00
EFB-02	3.04±0.5	2.06±0.8	3.0±0.5
EFB-03	9.0±0.6	6.05±0.7	8.02±0.3
EFB-04	0.5±0.7	0.2±0.1	0.5±0.5
EFB-05	00	00	00
EFB-06	2.0±0.4	1.05±0.9	3.02±0.3
EFB-07	00	00	00

Table 4.2: Zone of inhibition (mm) of Bioactive Metabolites (aqueous crude extracts) of Endophytic Bacteria in against different fungus.

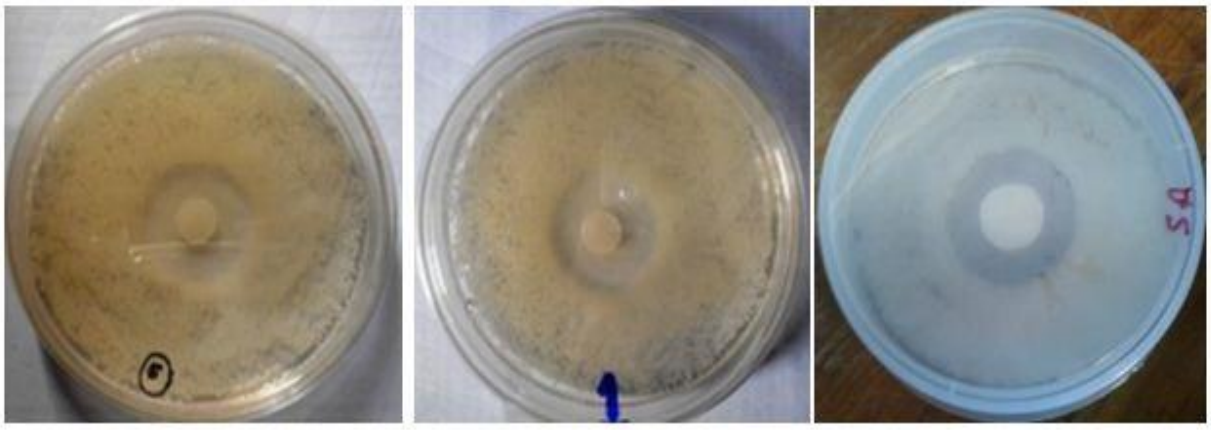

Figure 4.4: Anti-property of Bioactive Metabolites of Endophytes Bacteria (EFB-03) against different fungus.

Tested microorganisms were cultured on agar solidified medium. The 15 µl crude extracts from all 07 isolates were applied to the disk (6 mm diameter). The bacterial cultures were placed in an incubator at 37 ^{0}C and fungal culture at 28 ^{0}C for overnight incubation. After 24 hours, we observed the zone of inhibition against different bacteria and fungus and diameter of inhibition zone was measured. Out of all the 07 isolates, crude metabolites of an endophytic bacterial isolate (EFB-03) displayed significantly (maximum anti-property) antimicrobial activity against all pathogens; *B. subtilise, P. aeruginosa, E. coli, S. aureus, R. solanacearum, B. cereus, C. albicans, P. chrysogenum and P. exigua.* This result clearly indicates that extracts are quite effective against both gram positive and gram-negative bacteria as well as against plant and animal pathogenic fungi and bacteria. This showed that the metabolite of endophyte bacteria screened from root tissue yielded a broad-spectrum nature product that was used to show major antimicrobial activity against human pathogenic bacteria and human pathogenic fungi (*Candida albicans*). Apart from this, the anti-property was checked in against of 24 human pathogenic bacteria. These bacteria are as follows, *E. coli O157:H7, B cereus, B subtilis, B. megaterium, Staphylococcus aureus, Streptococcus pyogenes, Helicobacter pylori, B. megaterium, Multiple drug resistant S. aureus, Streptococcus pyogenes, Corynebacterium amycolatum, Chlamydia pneumonia, S. epidermis, Mycobacterium tuberculosis, Pseudomonas aeruginosa, Clostridium difficile, Klebsiella pneumonia, Corynebacterium diphtheria, Vibrio sp., Streptococcus pneumonia, Staphylococcus epidermidis, S. epidermis, Salmonella typhimurium, Salmonella typhi.* Against these various pathogenic bacteria, only the anti-property from bioactive metabolites of EFB-02 and EFB-03 were demonstrated.

Pathogenic Bacteria's	Diameter of inhibition zone in (mm)	
	EFB-02	**EFB-03**
E. coli O157:H7	18±0.8	20±0.8
B. cereus	1.01±0.8	4.0±0.7
B. subtilis	12±0.9	21±0.4
Megatherium	8.6±0.4	16.0±0.2
Staphylococcus aureus	06±0.5	19±0.6
Helicobacter pylori	07±0.21	14±0.8
Multiple drug resistant S. aureus	01±0.33	03±0.3
Streptococcus pyogenes	03±0.31	07±0.6
Corynebacterium amycolatum	08±0.1	14±0.5
Chlamydia pneumonia	12±0.4	17±0.6
S. epidermis	09±0.4	16±0.7
Mycobacterium tuberculosis	07±0.13	11±0.11
Pseudomonas aeruginosa	3.43±0.7	5.31±0.9
Clostridium difficile	10±0.51	14.50±0.83
Klebsiella pneumonia	16±0.31	20±0.32
Corynebacterium diphtheria	14±0.48	17.05±0.71
Vibrio sp.	09±0.64	11±0.11
Streptococcus pneumonia	12±0.25	16±0.63
Staphylococcus epidermidis	14±0.86	18±0.5

S. epidermis	14±0.84	18±0.51
Salmonella typhimurium	13±0.63	15±0.58
Salmonella typhi	09±0.49	11±0.17

Table 4.3: Antibacterial activity of Bioactive metabolites against different human pathogenic bacteria.

The results of the antimicrobial activity of the above 24 human pathogenic bacteria are given in Figure 4.5. Metabolites of the endophyte's bacterium (EFB-03) indicate the maximum zone of inhibition against the bacillus subtilis; it is the cause of various infectious diseases in humans. *E. coli O157: H7* (20±0.8) *which* is responsible for many diseases. In this experiment, the bioactive metabolite of EFB-02 was coded as 1 and the bioactive metabolite of EFB-03 is coded as 2 (figure 4.5).

Similarly, the fungi that have been tested are a high plant pathogenic fungus, the name of these fungi is as follows, *Sclerotium roflasi, Sclerotinia sclerium, Fusarium species*. The strong anti-property was showing bioactive metabolites of 03 endophytes bacteria against all these fungi. Therefore, these bacterial metabolites can be an important chemical remedy in checking the damage of crops. Based on this experimental result, it can be concluded that screening of a strong antibacterial compound from this isolation may provide a good alternative natural source as an antibiotic such as chloramphenicol fluconazole, etc.

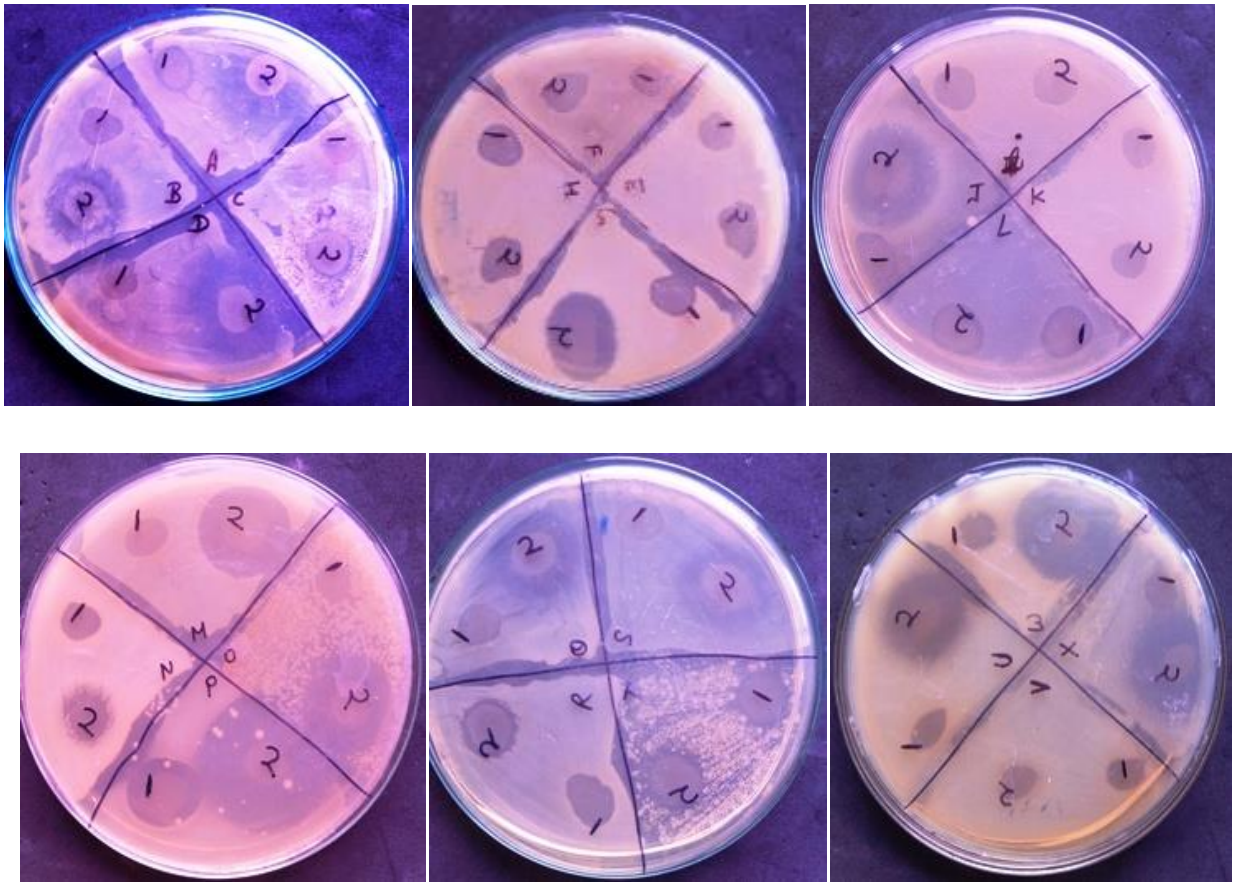

Figure 4.5: Bioactive metabolites of 2 strains (EFB-03 and EFB-06) were tested against 24 human pathogenic bacteria's (In each plate, we took 04 pathogenic bacteria).

The demand for the discovery of new antimicrobial agents is constantly increasing due to the development of pathogen resistance to available drugs at present. Many scientists are researching new synthetic drugs, but all these synthetic medicines are adversely affected by the environment. The only solution is that scientists should work on more and more natural medicines, and people should also use more and more natural medicines for different diseases.

4.1 Minimum Inhibitory Concentration (MIC): the MIC of crude extract of isolating EFB-03 that showed inhibitory effect was determined by dilution methods. The MIC values of crude extracts from isolated EFB-03 is shown in Table 4.4. The results indicated that the bacterial crude extract clearly showed antibacterial property against gram-positive, gram-negative bacteria and fungus. The extract obtained from isolates EFB-03 had MIC values of 240µg/ml, 150µg/ml, 200µg/ml, 250µg/ml, 200µg/ml and 140µg/ml for the *E. coli, B. subtilis, Penicillium chrysogenum, S. aureus, R. solanacearum* and *B. cereus* respectively.

Similarly, MIC value for test fungi was determined, which is as follows, the extract in this case had MIC value of 500µg/ml, 600µg/ml and 550µg/ml for *C. albicans, P. chrysogenum* and *P. exigua* respectively. Based on these results, we can suggest that EFB-03 producing high bioactive metabolites, i.e. Showing an excessive zone of inhibition against various bacteria and fungi, such bioactive compounds may be excellent compounds for further study. The MIC values of the group of bacteria which were tested in the department of microbiology, Institute of medical science Banaras Hindu University, Varanasi (IMS BHU). Different MIC values for bacteria and fungi showed major differences for bacteria and fungi group. Composition and the structural difference may be one of the possible reasons. The MIC value (Table 4.4) of crude extracts for both bacterial and fungal species explained that the molecules present in crude extract could be a good source of antimicrobial metabolites which can be effectively applied against human and plant pathogenic microorganisms. Therefore, further work should be done to explore the structure and biological properties of bacterial bioactive metabolite.

The minimum inhibitory concentration (MIC, µg/ml) of the crude metabolites produced by isolating EFB-03 which displayed the stronger antibacterial activity.			
Bacteria	**MIC (µg/ml)**	**fungi**	**MIC (µg/ml)**
E. coli	240 µg/ml	*C. albicans*	500 µg/ml
B. subtilis	150 µg/ml	*P. chrysogenum*	600 µg/ml
Penicillium chrysogenum	200 µg/ml	*P. exigua*	550 µg/ml
S. aureus	150 µg/ml		
R. solanacearum	200 µg/ml		
B. cereus	140 µg/ml		

Table 4.4: The minimum inhibitory concentration (MIC) of the crude metabolites against different bacteria and fungus produced by isolate EFB-03.

4.2 Morphological Identification of Isolate EFB-03: Endophytic bacteria were isolated from the healthy root of Adhatoda beddomei. Identification of bacterial species was done on the basis of cultural and morphological characteristics features like the appearance of the colony, colony colour, texture, and margins, as well as microscopic observations, such as the size of cells and their arrangements, were examined for species differentiation. Bacterial species colonized on the nutrient agar media (NAM) and the shape of cells are usually between cocci and rod.

Microscopic (scanning of electron microscope) studies of the bacteria isolate has shown cells surfaces slightly rough, cell mass is grey and slightly black and size of bacterial cells is 8.5µm (Raper and Fennell, 1965; Klich, 2002).

Morphological characteristics have been shown in following given figures below (Figure 4.6). The Based on these typical features, the bacteria (EFB-03) have been partial identified.

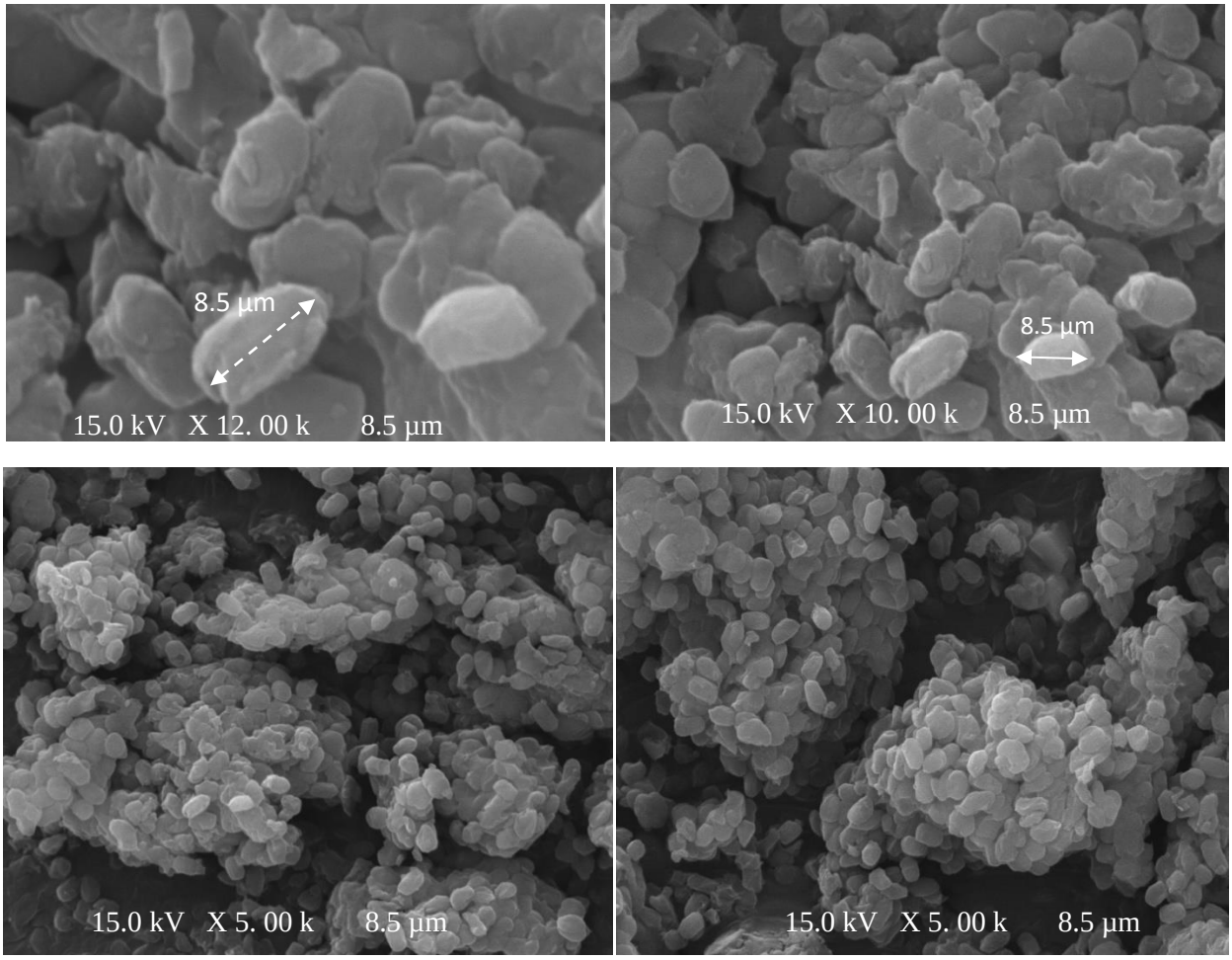

Figure 4.6: Image of endophytes bacteria (EFB-03) taken by scanning electron microscope (SEM).

4.3 Media optimization:

4.3.1 Effect of different carbon sources for the production of bioactive metabolites: To optimize the production of antimicrobial agent, the experiments were performed using different carbon, nitrogen, temperature, incubation period, different carbon and nitrogen concentrations were varied for optimizing media. Because of all these parameters, only one endophytic bacterium (EFBG-03) was used to achieve maximum production of bio-metabolism.

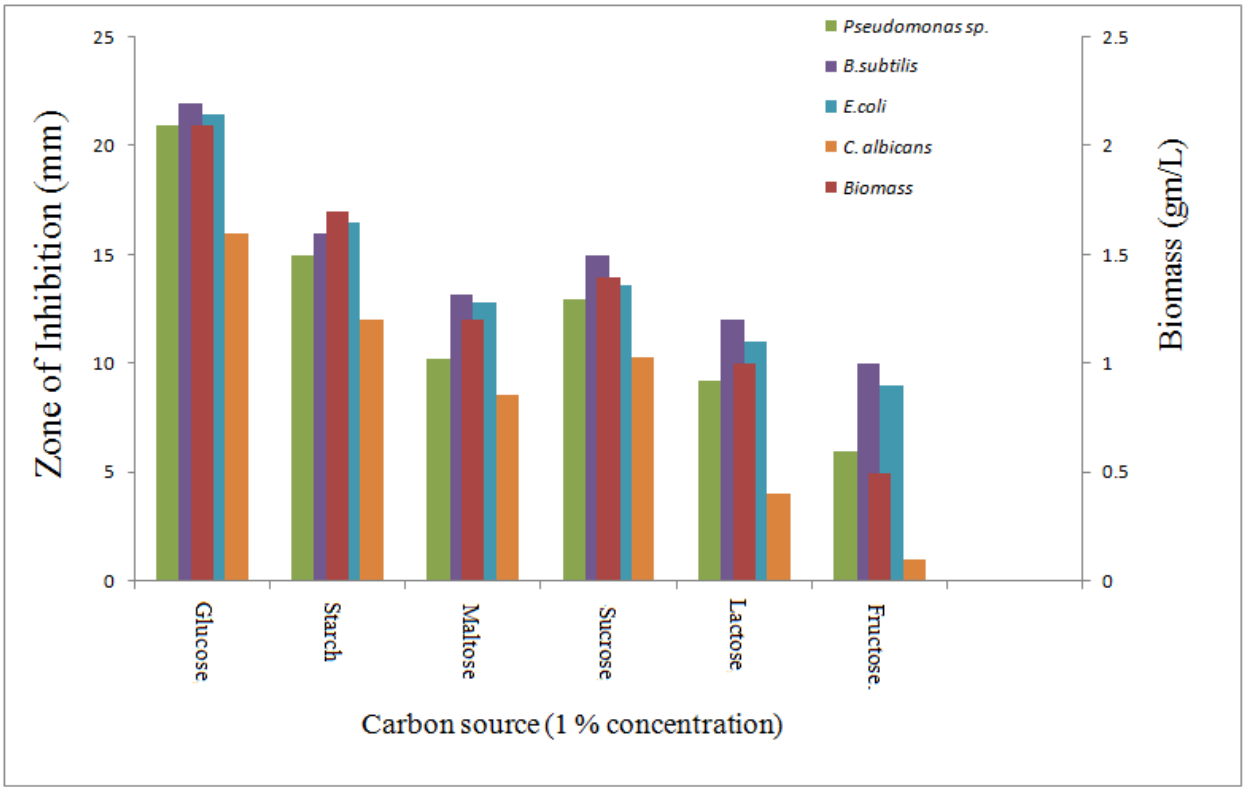

Figure 4.7: Effect of different Carbon sourceson for the production of bioactive metabolites.

Various carbon sources such as glucose, starch maltose, sucrose, lactose, and fructose were used in these experiments.

Production of different levels of bioactive metabolites was found using various carbon sources. During this experiment, each carbon source was taken as a concentration of 1% (w/v) and each carbon was kept at the same level. All carbon sources and endophytes inoculum (EFB-03) were added to the production media. And was kept for 3 days to incubate. After the end of the incubation period, (figure 4.7) it was found that glucose is an excellent carbon source under which endophytes bacteria were producing the maximum bioactive metabolites. The bioactive metabolites produced in the presence of glucose were bioactive metabolites showing the maximum zone of inhibition against various bacteria and fungi. Achieving the maximum area of inhibition against various bacteria and fungi by bioactive metabolites means that the maximum production of bioactive metabolites is occurring, and it also shows that the maximum formation of cell biomass (2.2 gm/L) also occurs in the presence of glucose. Production of bioactive metabolites and cell biomass formation was found to be very poor in the presence of fructose. Because the bioactive metabolites produced in the presence of fructose received very little zone of inhibition against various bacteria and fungi and there is also a minimum formation of cell biomass (0.6 gm/L). The production of bioactive metabolites and the formation of cell biomass in the presence of fructose during the whole experiment is happening at a very bad level, so it can be said that fructose is acting as a bad carbon source for this experiment. And starch and sucrose are showing for the production of bioactive metabolites and cell biomass formation at an intermediate level.

4.3.2 Effect of different Glucose concentration on the production of bioactive metabolites: In this experiment, various glucose concentrations were used for the production of maximum bioactive metabolites and cell biomass formation. The glucose concentration of 15gm/L showed remarkable results followed by 20 gm/L and 22.5 gm/L concentrations. At 1.5% concentration of glucose get maximum bioactive metabolite production and formation of cell biomass. The

bioactive metabolites produced in the presence of 1.5% glucose concentration give a maximum zone of inhibition against different bacteria (The 23 mm zone of inhibition is that of *B. subtilis*) and fungi (17mm zone of inhibition is *C. albicans*). And at 0.5 and 2.25% concentration of glucose, very little bioactive metabolite was produced and cell biomass was formed. In this way the concentration of 1.5% glucose in this whole experiment is considered as a good concentration for the production of maximum bioactive metabolites and cell biomass formation.

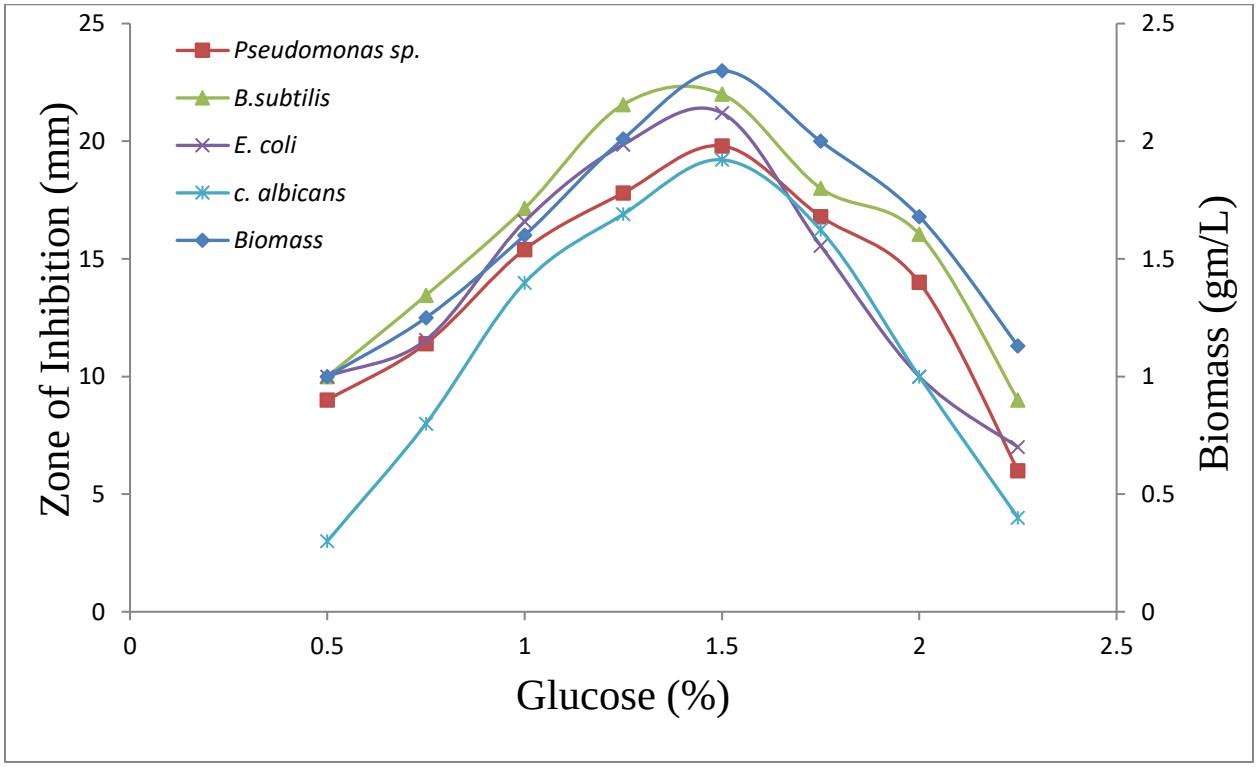

Figure 4.8: Production of bioactive metabolites and formation of cell biomass at various glucose concentrations.

4.3.3 Effect of different Nitrogen sources for the production of bioactive metabolites: The effect of various Nitrogen sources was also studied for the production of bioactive metabolites. The nitrogen sources used in the studies is, KNO_3, $NaNO_3$, NH_4Cl, NH_4NO_3, Yeast extract, peptone and Beef extract. Each nitrogen source was taken at 0.3 % (w/v). From these studies, sodium nitrate (NaNO3) is the best nitrogen source for enhanced production of antimicrobial metabolites, followed by potassium nitrate and ammonium chloride (Figure 4.9). Different microbes require different nitrogen sources for growth and development.

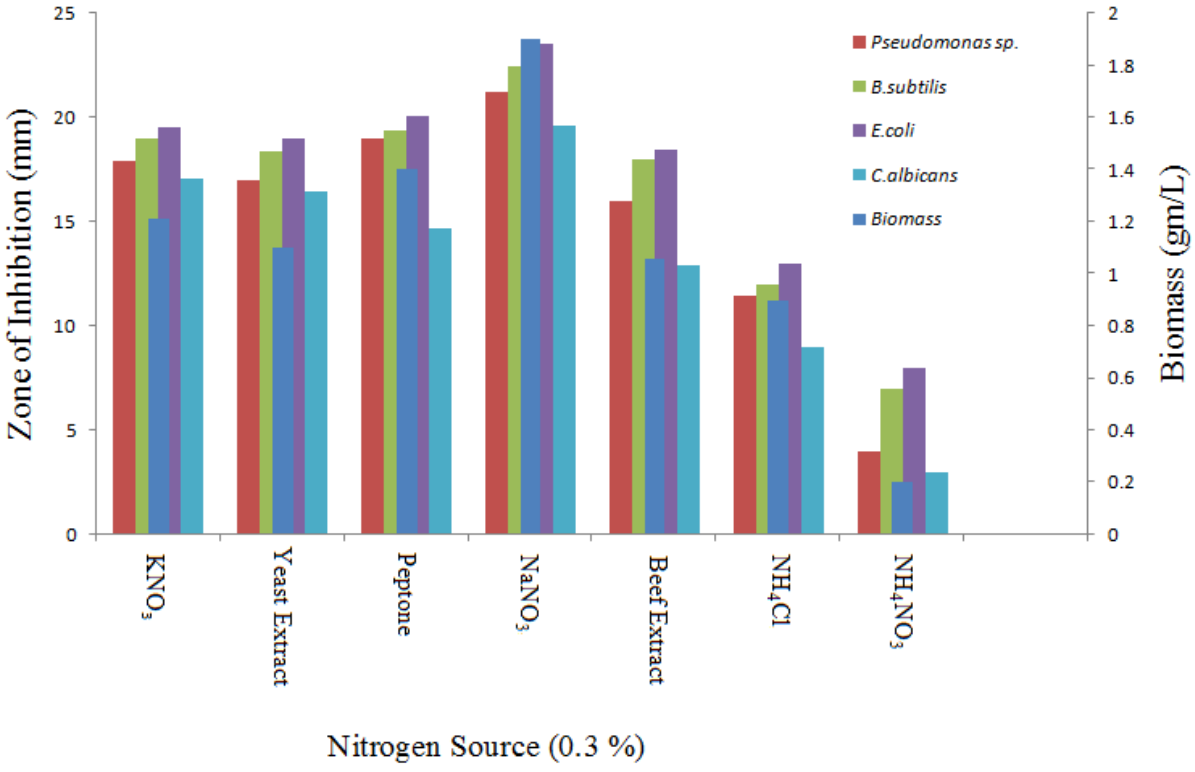

Figure 4.9: Effect of different nitrogen sources on antimicrobial metabolites production.

In the case of most microorganisms, both inorganic and organic nitrogen sources are required to produce protein, nucleic acids, and various cell components. But it has also been observed that some carbon and nitrogen sources have an inhibitory effect in antimicrobial agent production. The EFB-03 isolated strain of endophytes bacteria was used in this study. EFB-03 isolated strain is producing maximum bioactive metabolites (obtained the maximum zone of inhibition against *B. subtilis* and *E. coli*) in the presence of $NaNO_3$, in the presence of NH_4NO_3 bioactive metabolites were produced at very poor levels. Whereas in the presence of beef extract, peptone, and yeast extract, endophytes bacteria (EFB-03) produced bioactive metabolites up to certain-level.

4.3.4 Effect of different $NaNO_3$ concentration on the production of bioactive metabolites and cell biomass formation: During these studies, observing the effect of varying NaNO3 concentrations, it was found that the bio-concentrations of which play an important role, directly or indirectly, in the production and formation of cell biomass. In this set of experiments, Various concentrations {0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 (% w/v)} of $NaNO_3$ were used.

At a concentration of 0.5% of $NaNO_3$ the maximum zone of inhibition (maximum zone inhibition means maximum bioactive metabolites production and cell biomass formation) was observed against *B. subtilis and E coli (*Figure 4.10). At 0.5% concentration of $NaNO_3$, maximum bioactive metabolites were produced and cell biomass were formed. Whereas at 0.2, 0.3 and 0.6% concentrations of NaNO3, endophytes bacteria were producing bioactive metabolites up to mid-level. The production of minimal bioactive metabolites was found at a 0.7% (w/v) concentration of NaNO3.

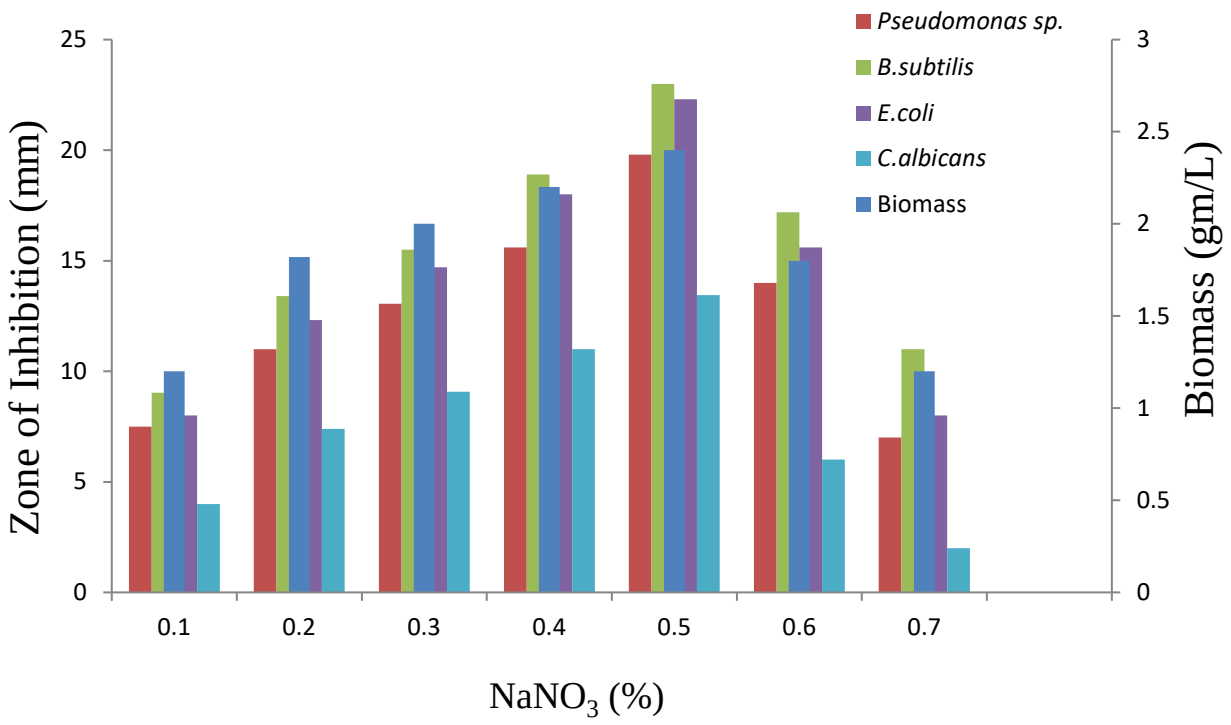

Figure 4.10: Effect of various concentration of $NaNO_3$ on production of antimicrobial metabolite.

4.3.5 Effect of different pH for the production of bioactive metabolites and cell biomass formation: During this study, various pH media were used to produce bioactive metabolites. The production of bioactive metabolites and cell biomass formation was obtained with different concentrations on different pH. On some pH, the production of bioactive metabolites and the formation of cell biomass was received in good concentration. Initial pH 7.0 of the medium was observed to be optimal for growth and bioactive metabolites production by EFB-03 (figure 4.11). The maximum zone of inhibition in a way indicates the production of maximum bioactive metabolite and cell biomass formation. (2.3 gm/L). Bioactive metabolites of pH 7.0 showed maximum zone of inhibition (22, 23 mm in diameter) in against *E. coli* and *B. subtilis*. Although initial pH 4.0, 5.0, 9.0 and 10.0 also supported to biomass formation and bioactive metabolite

production but the lower yield was observed. No concentration of cell biomass and bioactive metabolites was obtained on this pH (03, 11, and 12). Digrak et al., (2001) It was reported by Metin Digrak that the highest production of biomass was at pH 7.5. While the production and formation of bioactive metabolites and cell biomass at pH 11, 12 was negligible. The reason for this is that during the production process of bioactive metabolites, there is also the formation of an inhibitory product (Mohos et al., 2019). The pH of culture media is one of the very important determining parameters for the metabolism and biosynthesis of bioactive metabolites. According to Hansen and Wang, Permeability characteristics of the cell wall and membrane are also related to the cell environment pH value and effected on either ion uptake or loss of the nutrient medium.

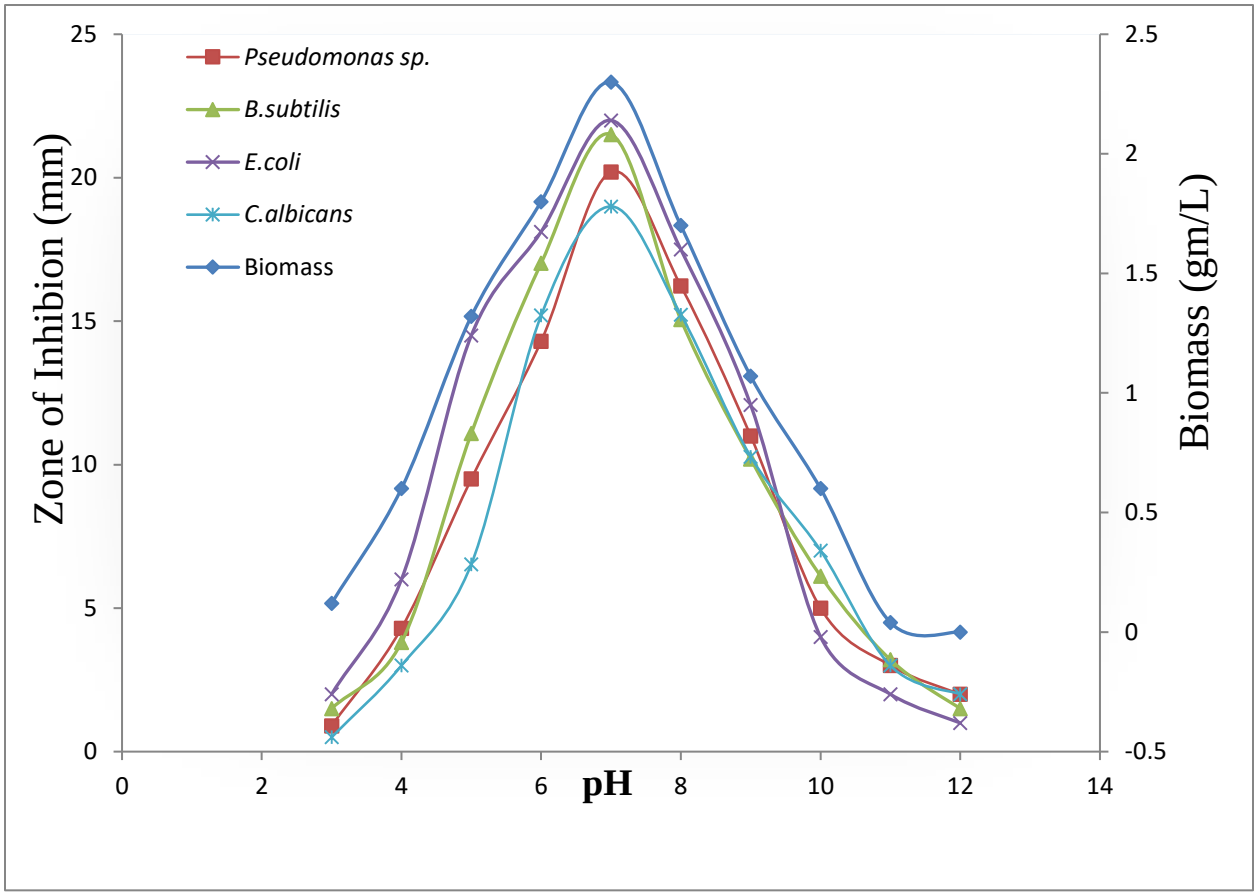

Figure 4.11: Effect of initial pH on antimicrobial bioactive metabolites production.

4.3.6 Effect of different incubation temperature (^{0}C) on the production of bioactive metabolites and cell biomass formation: Different temperatures (20 to 06 ^{0}C) were used in these studies. The incubation temperature directly affects the overall growth and development pattern of the endophyte's bacteria and subsequently, and also plays its important role in the synthesis of various metabolites. The productions level of bioactive compounds was observed at different temperatures like 20 ^{0}C,25 ^{0}C, 30 ^{0}C, 35 ^{0}C, 40 ^{0}C, 45 ^{0}C, 50 ^{0}C, 55 ^{0}C and 60 ^{0}C by EFB-03. Maximum growth of cell biomass and bioactive metabolite production by isolated EFB-03 strain were recorded on incubation temperature 35 ^{0}C (±2) and it was followed by 40^{0}C, 45^{0}C, respectively (Figure 4.12). The maximum zone of inhibition indicates maximum bioactive metabolite production and cell biomass formation (2.4 gm/L). Bioactive metabolites of incubation temperature 35 0C (±2) are evidence for the maximum zone of inhibition (21, 22 mm in diameter) against *E. coli* and *B. subtilis*.

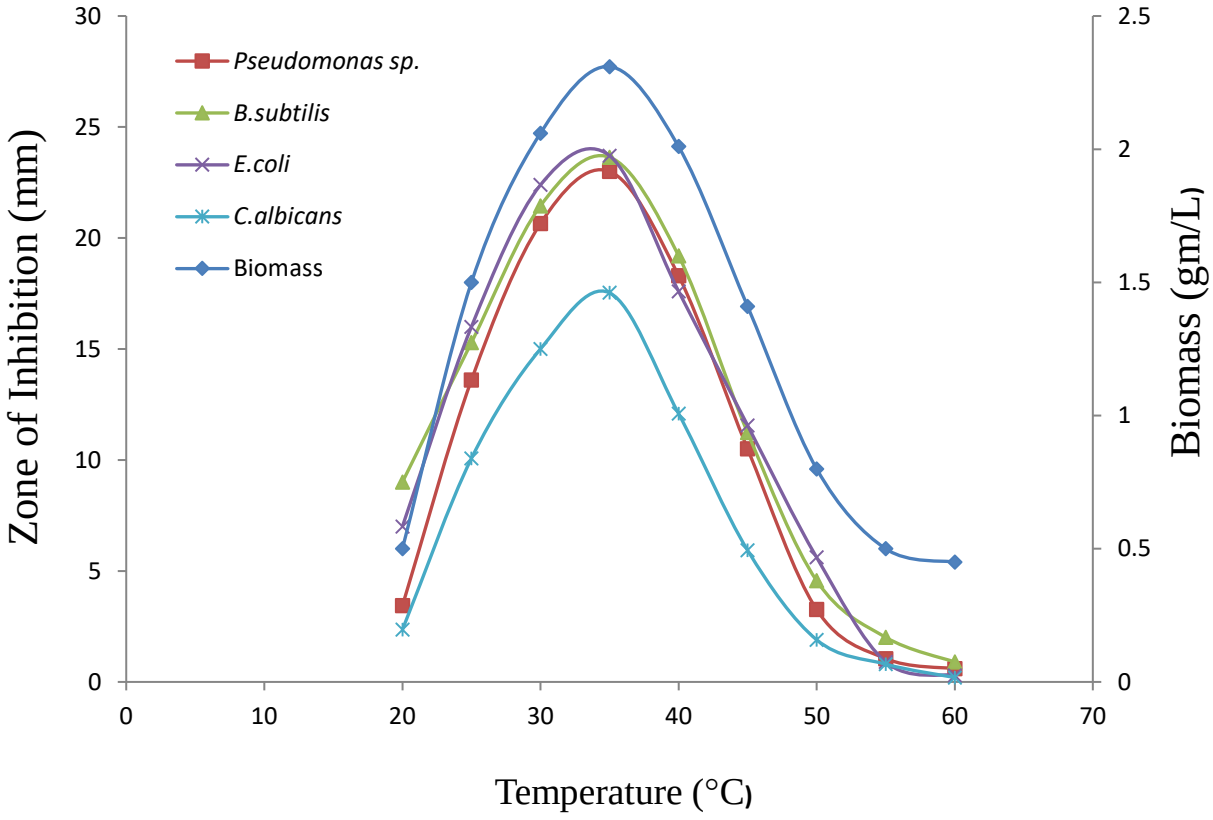

Figure 4.12: Effect of different incubation temperature on production of antimicrobial metabolite.

The production of bioactive metabolites and cell biomass formation was become negligible at a temperature of 20 ^{0}C, 50 ^{0}C, 55 ^{0}C, and 60 ^{0}C.

4.3.7 Effect of different incubation periods on the production of bioactive metabolites and cell biomass formation: Based on these experiments, in various incubation periods, the level of production of bioactive metabolites and cell biomass formation of EFB-03 were checked. Different amounts of bioactive metabolites and cell biomass were obtained based on different incubation periods. the maximum production of bioactive metabolites and cell biomass formation was achieved on the third day (Figure 4.13). The bioactive metabolites of third day were indicating a

maximum zone of inhibition (means maximum production of bioactive metabolites and cell biomass formation) towards *B. subtilis*.

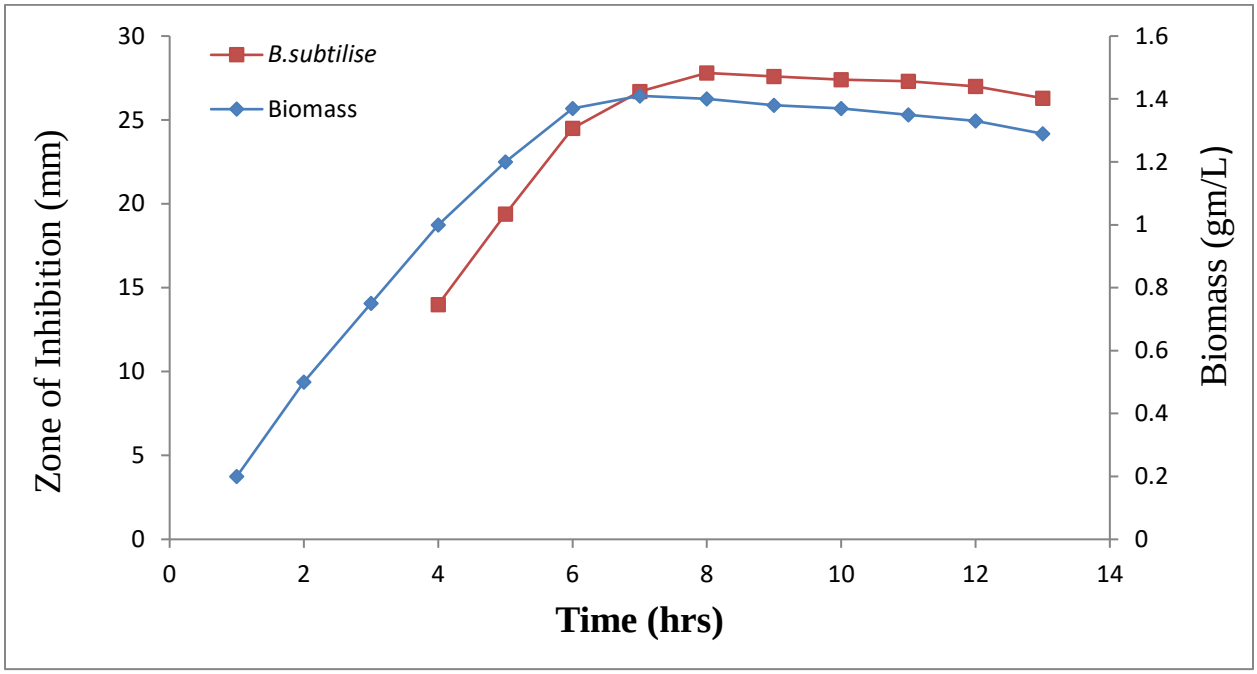

Figure 4.13: Effect of different incubation period on antimicrobial metabolites production.

In this experiment, the level of production of bioactive metabolites and cell biomass formation remains the same for a few days, but after a few days, it was continuously decreasing. From endophytes bacteria (EFB-03), maximum bioactive metabolites and cell biomass were obtained on the third day.

4.4 Response surface methodology: Microbial bioactive metabolites produced by endophytes bacteria (EFB-03) were increased by optimization of the medium components through central composite design (CCD) of response surface methodology (RSM). Two or Three- experiments were conducted in triplicate with different carbon, nitrogen, some's pH and temperature (Table 4.5). The response obtained from experiments of central composite design were calculated with second-order polynomial multiple regression. In the case of bioactive metabolite production from endophytes bacteria (EFB-03), the parameters nitrogen and carbon concentration exerted the most significant effect. These three sets of experiments yielded an average biomass production of 3.18gm/L at pH 6.89 temperature 29.49 ^{0}C carbon concentration 2.298 gm/L and nitrogen concentration 0.29 gm/L after 03 days of incubation. These experiments, it was observed that the experimental adequacy of the model and the existence of the optimal point were correctly confirmed by a good agreement between the predicted and experimental results. The regression model was considered to demonstrate the joint effect of the independent variable and the collective effect of the individually independent variable on the response variable. The response surface interpreting the quadratic effect of pH and temperature on bioactive metabolite production and cell biomass formed by endophytes bacteria (EFB-03) is shown in Figure 4.14 A, B, C, D, E and F, In the case of bioactive metabolite production and cell biomass formation from EFB-03, all the parameters nitrogen and carbon concentration exerted the most significant effect. The cell biomass model showed F-value conforming to 3.023 indicating the significance of the model with a p-value of <0.022 (Table 4.5).

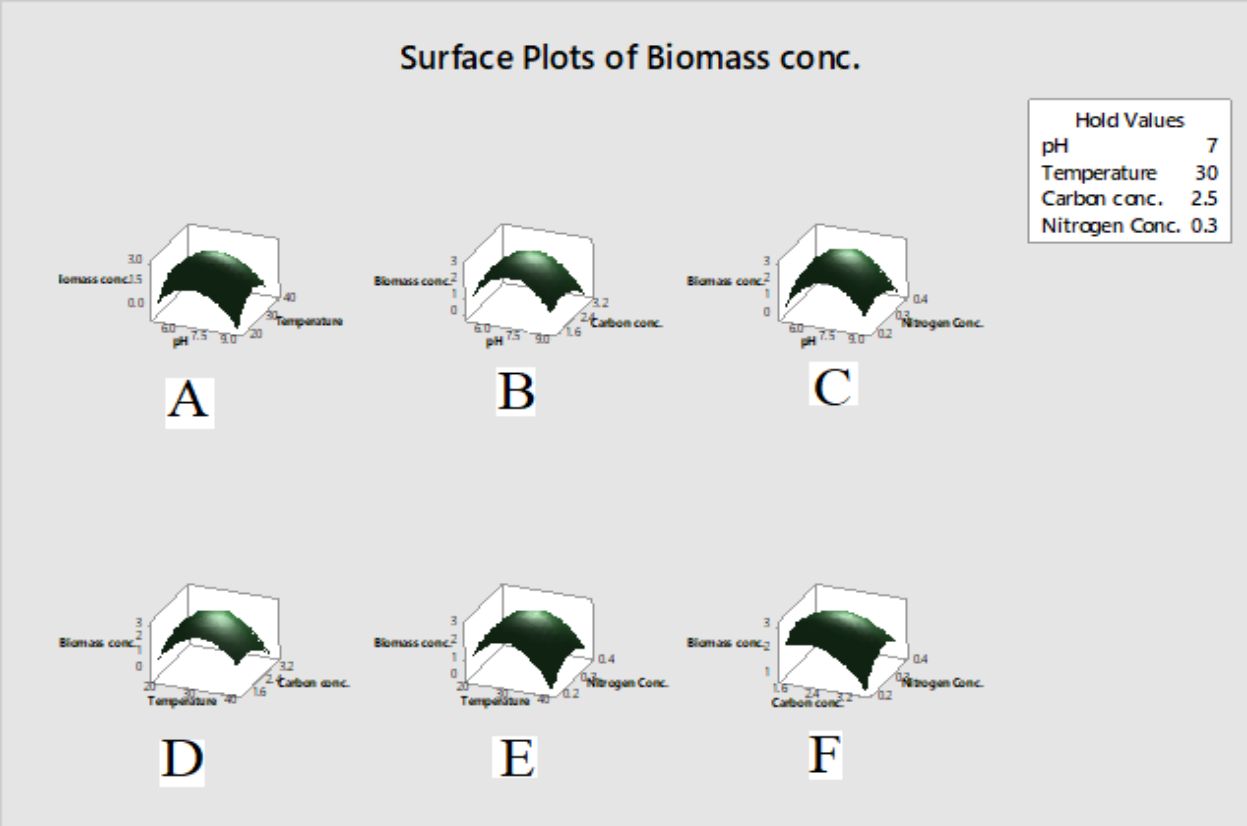

Figure 4.14: Response surface plots (3-dimensional view) showing interactive effects of selective variables on cell biomass formation.

A: Effect of pH and Temperature on the Cell Biomass formation using Endophytes bacteria (EFB-03). **B:** Effect of pH and carbon concentration on the Cell Biomass formation using Endophytes bacteria (EFB-03). **C:** Effect of pH and nitrogen concentration on the Cell Biomass formation using Endophytes bacteria (EFB-03). **D:** Effect of Temperature and carbon concentration on the Cell Biomass formation using Endophytes bacteria (EFB-03). **E:** Effect of Temperature and nitrogen concentration on the Cell Biomass formation using Endophytes bacteria (EFB-03). **F:** Effect of carbon and nitrogen concentration on the Cell Biomass formation using Endophytes bacteria (EFB-03).

From this analysis, it shows that the models chosen explain, the relationship between factors and response correctly. Further, the Adjacent R-squared value of 3.18g/L was found to be very close to the pre-R-squared value of 3.07g/L indicated that the model was suitable and can be used for the quantification of cell biomass formation.

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	3.023	0.140	21.57	0.000	
Blocks					
1	0.1695	0.0657	2.58	0.022	1.00
pH	-0.1067	0.0692	-1.54	0.46	1.00
Temperature	-0.1192	0.0692	-1.72	0.107	1.00
Carbon conc.	-0.1642	0.0692	-2.37	0.033	1.00
Nitrogen Conc.	-0.0350	0.0692	-0.51	0.021	1.00
pH*pH	-0.4375	0.0648	-6.75	0.000	1.05
Temperature*Temperature	-0.4425	0.0648	-6.83	0.000	1.05
Carbon conc.*Carbon conc.	-0.1862	0.0648	-2.88	0.012	1.05
Nitrogen Conc.*Nitrogen Conc.	-0.2087	0.0648	-3.22	0.006	1.05

pH*Temperature	0.0662	0.0848	0.78	0.448	1.00
pH*Carbon conc.	-0.0475	0.0848	-0.56	0.584	1.00
pH*Nitrogen Conc.	-0.0812	0.0848	-0.96	0.354	1.00
Temperature*Carbon conc.	-0.1413	0.0848	-1.67	0.118	1.00
Temperature*Nitrogen Conc.	0.0750	0.0848	0.88	0.391	1.00
Carbon conc.*Nitrogen Conc.	0.0812	0.0848	0.96	0.354	1.00

Table 4.5: Analysis of variance (ANOVA) for response surface quadratic model obtained from experimental designs.

The quadratic model was found to be significant according to the sequential model sum of the squares (p-value <0.0001). The lack of fit test values from the sequential model is appropriate for the quadratic model, illustrating two significant effects of fit. Adjusted R^2 and Predicted R^2 for this quadratic model came out as the finest model. Finally, the complete quadratic polynomial model in terms of the obtained normative factors is:

Biomass concentration = -45.42 + 6.35 pH + 0.997 Temperature + 4.78 Carbon conc.

+ 43.7 Nitrogen Conc. - 0.4375 pH*pH - 0.01770 Temperature*

Temperature- 0.745 Carbon conc.* Carbon conc. - 83.5 Nitrogen Conc.*

Nitrogen Conc.+ 0.0133 pH* Temperature

- 0.095 pH*Carbon conc.- 1.63 pH*Nitrogen Conc.- 0.0565 Temperature*

Carbon conc. + 0.300 Temperature* Nitrogen Conc.

+ 3.25 Carbon conc.* Nitrogen Conc.

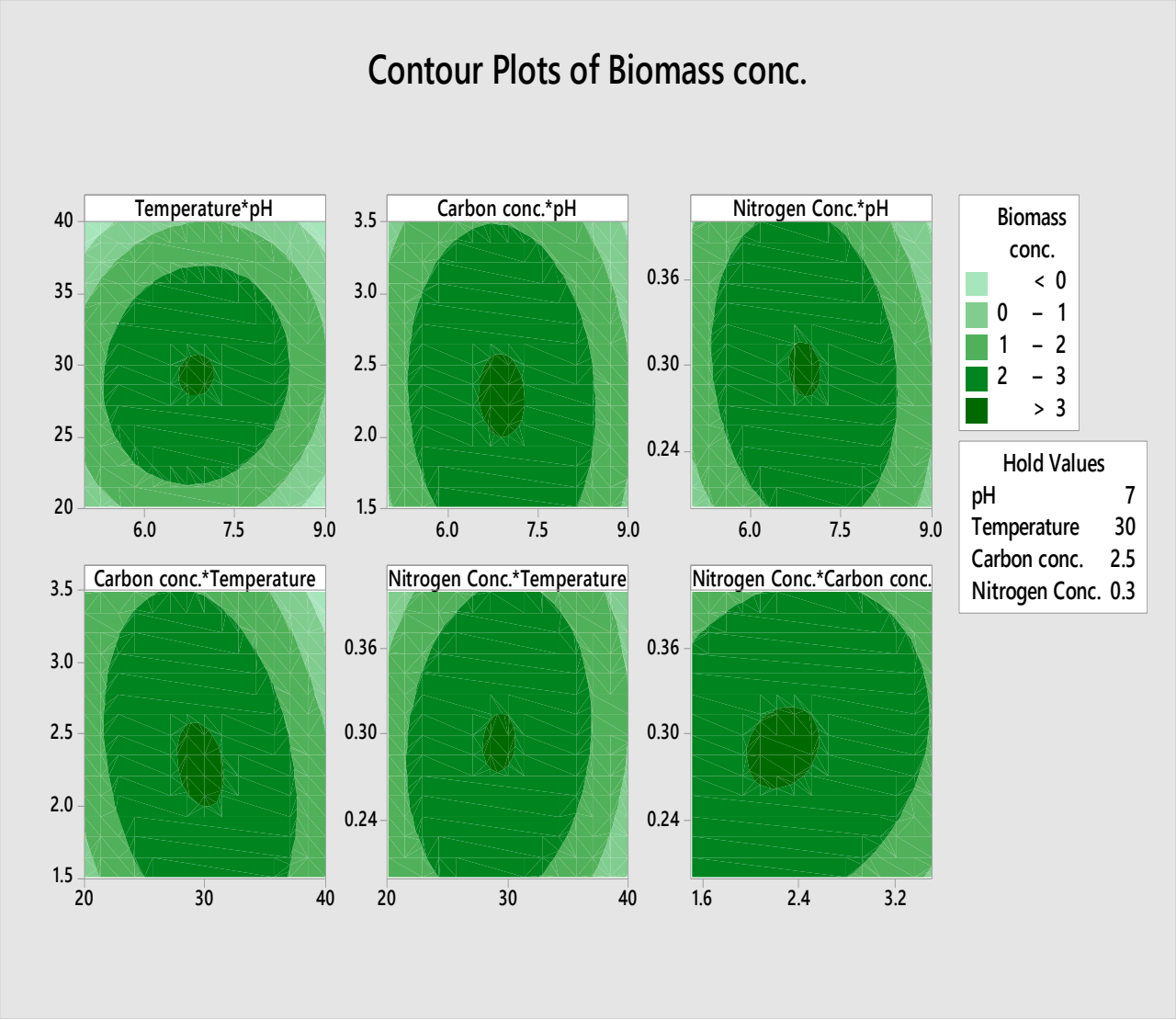

Figure 4.15: Contour plots of cell biomass formation of Endophyte bacterial isolate.

S. No	pH	Tempt.	Carbon. Con.	Nitrogen. Con.	Bio Mass	FITS1
1	6	25	2.0	0.25	2.80	2.29550
2	8	28	2.0	0.25	2.40	2.20717
3	6	35	2.0	0.25	2.10	2.05717
4	8	35	2.0	0.25	2.00	2.23383
5	6	25	3.0	0.25	1.98	2.18217
6	8	25	3.0	0.25	1.90	1.90383
7	6	35	3.0	0.25	1.50	1.37883
8	8	35	3.0	0.25	1.40	1.36550
9	6	25	2.0	0.35	1.90	2.07550
10	8	25	2.0	0.35	1.80	1.66217
11	6	35	2.0	0.35	2.40	2.13717
12	8	35	2.0	0.35	2.05	1.98883

13	6	25	3.0	0.35	2.78	2.28717
14	8	25	3.0	0.35	1.50	1.68383
15	6	35	3.0	0.35	1.45	1.78383
16	8	35	3.0	0.35	1.20	1.44550
17	7	30	2.5	0.30	3.00	3.19300
18	7	30	2.5	0.30	3.10	3.19300
19	7	30	2.5	0.30	3.08	3.19300
20	7	30	2.5	0.30	3.08	3.19300
21	5	30	2.5	0.30	1.02	1.31733
22	9	30	2.5	0.30	1.07	0.89067
23	7	20	2.5	0.30	1.00	1.32233
24	7	40	2.5	0.30	1.05	0.84567
25	7	30	1.5	0.30	2.10	2.43733
26	7	30	2.5	0.20	1.92	2.08900

27	7	30	2.5	0.40	2.00	1.94900
28	7	30	2.5	0.40	2.02	1.94900
29	7	30	2.5	0.30	3.10	2.85400
30	7	30	2.5	0.30	3.08	2.85400

Table 4.6: Experimental design and results of optimization of nutrient medium for the formation of cell biomass of Endophytes bacteria (EFB-03) by the central composite design.

The model p-Value (<0.0001) for the ANOVA analysis, coefficient of determination (R^2 =3.18) and adjusted coefficient of determination (adjusted R^2 =3,07), the quadratic polynomial model is highly significant and can be used to exhibit the relation between response and the significant variables as shown on the supplemental. This model is valuable to assess the direct collaboration and the quadratic effects in optimizing the parameters for increasing the bioactive metabolite production and cell biomass formation.

4.5 Column Chromatography: during the column chromatography technique, seventeen fractions at >4hrs periods were collected by column chromatography technique and the most active fractions against the pathogen range from F-8 to F-14 respectively. There were also some fragments in which there was no anti-property against different microbes (F-01 to F-06). A total of 17 fractions of bacterial semi- solid bioactive metabolites were tested. Also, the purification of the compound (fractions) was also confirmed by TLC.

4.6 Purification of crude extract by TLC: Before to structural elucidation, TLC was performed by using methanol ethyl acetate and chloroform to ensure the purity of the seven fractionated bioactive metabolites (compounds), Which was showing more anti-property against various microbes.

Maximum TLC is used to identify compounds (bioactive molecules) in the mixture, it compares the Rf value of an unknown compound to the Rf value of a known compound. Exhibited that the spots on the plates were developed and observed Under UV light at 254 nm and in this, no visualizer was used because the bacterial bioactive metabolites were already light brown and dark-colored compounds. Different retention factors (Rf) was obtained from different fractions bioactive metabolites samples by TLC.

Different band corresponding to a Rf value (0.35, 0.34, 0.67, 0.54, 0.56, 0.71, and 0.52) were showed considerable effective activity against the fungal and bacterial-pathogen. Methanol, ethyl acetate and chloroform extracts of bioactive metabolites have shown the maximum of 02 bands (**Figure 3.4)** TLC was performed to find out the number of compounds (bioactive) from fractionated metabolites (after column chromatography). It has to be checked on how many compounds are present in each fraction.

SPECTROSCOPIC ANALYSIS OF COMPOUNDS: After column chromatography and TLC, the various characterizations have done to get the correct structure of different fractions. In which NMR, MASS, FTIR, HPLC and UV- visible spectroscopy were considered.

4.7 Structural characterization of bioactive metabolites:

4.7.1 ^{1}H and ^{13}C of Compound 01: This compound is dark brown in color. ^{1}H NMR (dmso-d6,300 MHZ) spectra show one alkane group attached to a primary carbon atom by exhibiting a singlet at δ 0.805. 1.96 to 3.38 chemical shift indicates to the two other carbonyl groups exhibiting an overlapped doublet (d). 02 protons under oxygen residue by showing a signal at δ 3.42 and is located at C16 and C18. And a particular peak range (3.20 to 3.98) was observed, which denotes the existence of alcohol. The rest of the signal between δ 4.08 and 7.98 are due to phenol protons.

Another peaks range (8.12 to 8.18 chemical shift) of the aromatic groups is seen in the graph of ^{1}H NMR. 4.38 (m, 2H), 4.08(m,1H), 7.10-7.90 (m,4H- - NH_2), 1.96 (t,4H), 1.33(t, 2H), 1.23 (m,10H), 1.71(m,12H), 5.62-5.7 (m,4H),4.38 (s,1H), 0.805(d,3H).

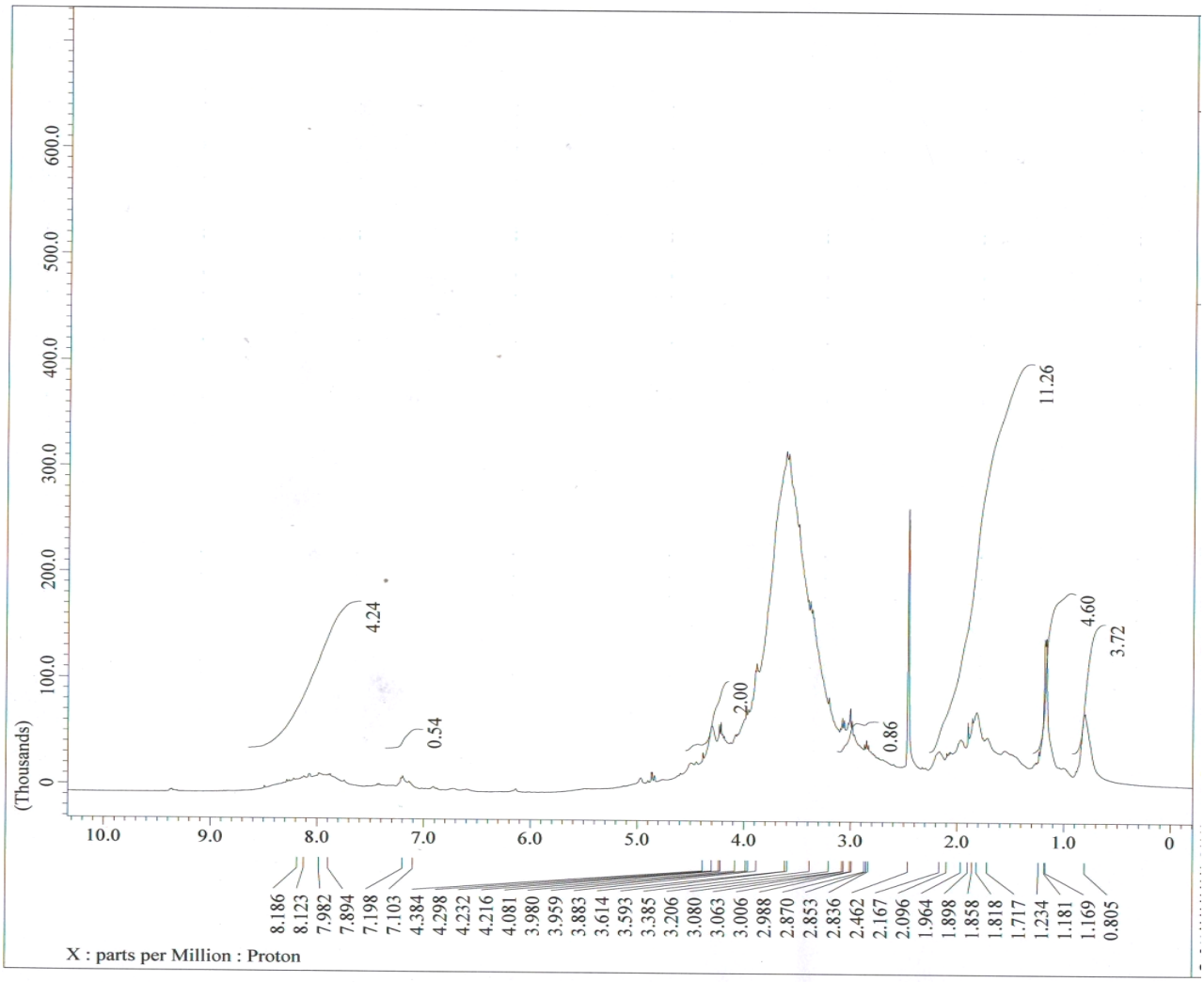

Figure 4.16: ^{1}H NMR spectrum of the compound 1 from isolated endophyte bacteria.

When performed ^{13}C for Compound 01, Some peaks are found as follows, ^{13}C-NMR spectra confirm the presence of the two-alcohol group (C-OH) by exhibiting signals at λ 60.23ppm and 61.65ppm and the signal at λ 40.143ppm to 40.478ppm indicate the existence of the three aldehydes group (C-C=O). The signal at λ 164.46ppm shows the presence of a carbonyl group as shown in figure 4.17.

In this graph, four alkanes (C-CH_2) are obtained between λ 34.4ppm to 39.9ppm, respectively. And together, a group of alkanes (C-CH_3) and aromatic (C = C) are also found at the peaks λ 14.94ppm and 133.49ppm.

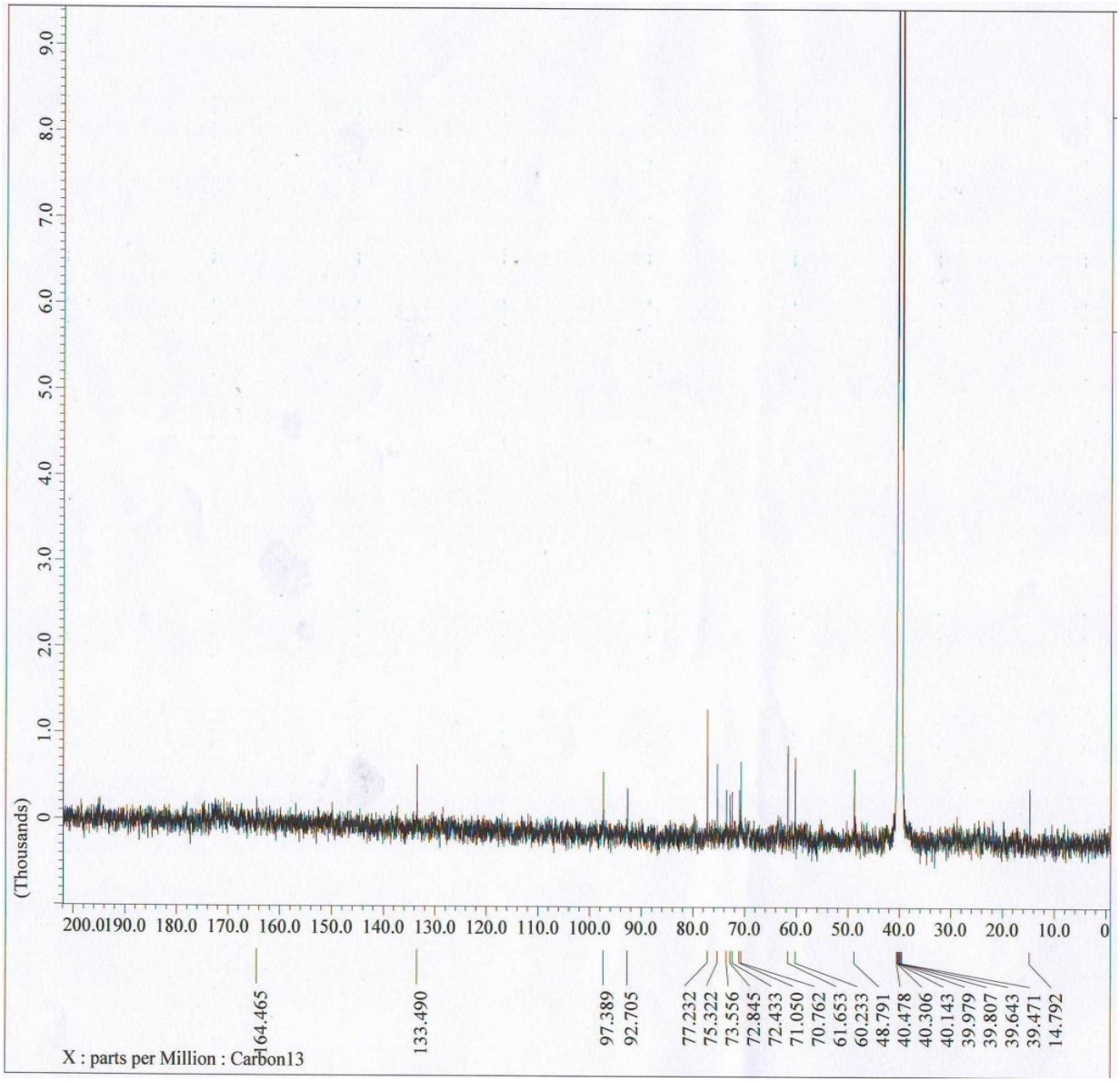

Figure 4.17: ^{13}C NMR spectrum of the isolated compound 01 from endophytes bacteria.

4.7.2 Mass Spectrum analysis of compound 01:

The mass spectrum implies that this fragment of bioactive metabolites can support the following structure. When performed mass spectrophotometer of Fragment 01, a particular molecular weight (423.5) was found. From ESI-MS m/z 423.5, 437.8 and 449.8 grams/mole fragments that confirm the presence of dispersed chain hydrocarbon units.

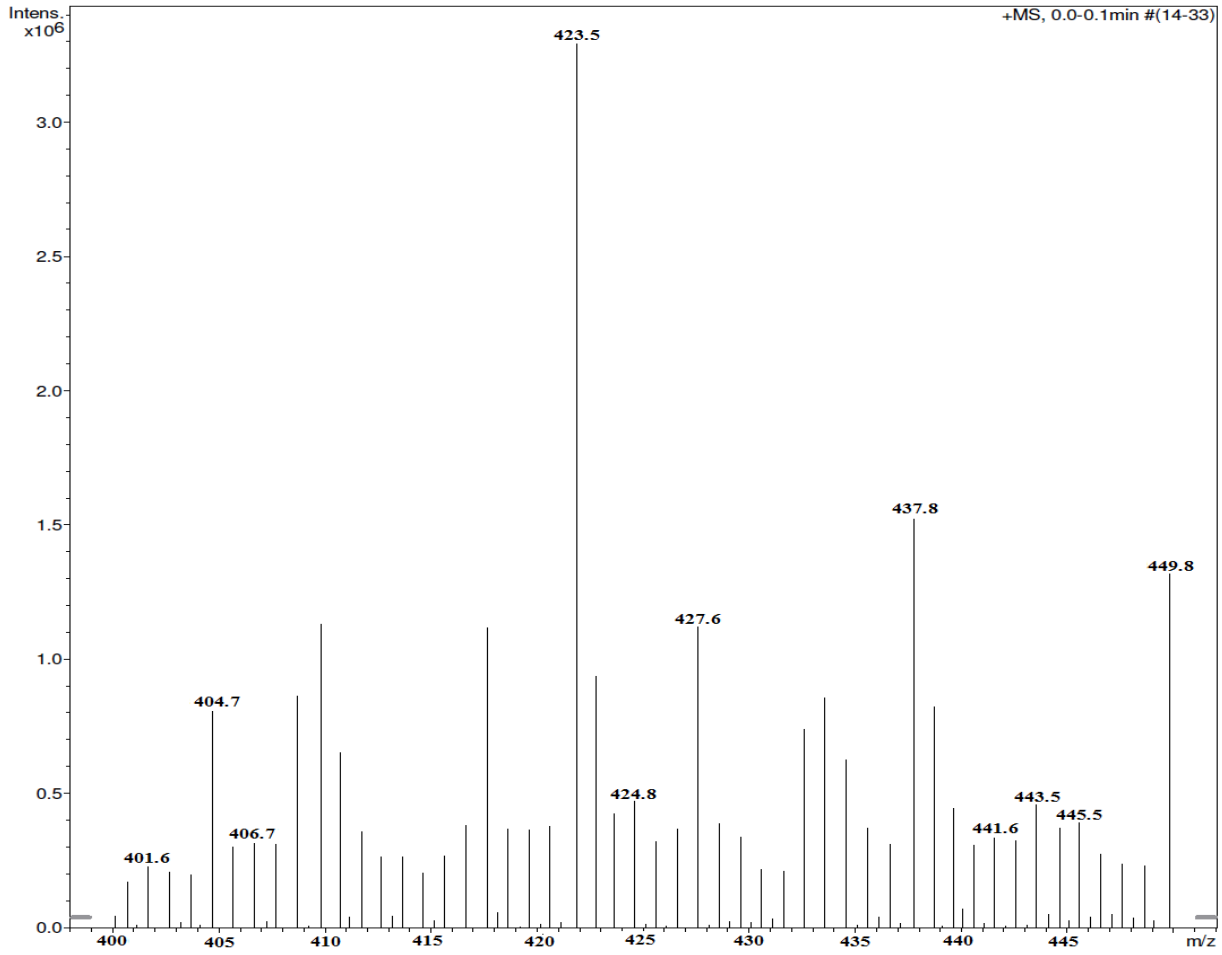

Figure 4.18: Mass spectroscopy analysis of compound 01.

4.7.3 Fourier transform infrared (FT-IR) spectroscopy of compound 01: Spectroscopy data analysis is a great help in obtaining the structure / chemical functionality of any bioactive compound and when it is run under the IR region. The weak absorption band of IR (KBr) υmax 773.46 cm^{-1} shows the presence of chloride in bacterial bioactive metabolites. The band between 1506.41 to 1558.48 cm^{-1} is generally indicated to the aromatic compounds. This O-H stretching specifies to the phenolic compound that has the best antimicrobial properties. And another pick was observed which is at 3728 cm-1, these picks is pointing towards phenol. Apart from this, there are some other peaks as well, such as 1856.99, 3649.32 cm-1 stretch which marks aromatic and alcohol groups respectively. Functional groups of isolated compounds from microbial bioactive metabolites are identified by FTIR spectroscopy. The FTIR data of the isolated compound from microbial bioactive metabolites are shown in the figure 4.19 and the compound showed absorption bands in the IR spectrum at 3628.10cm-1
for a OH group, 1456.76 cm-1 for C=C group, 1558.48cm-1 for C=N group.

S. No	Characteristic Absorption (cm-1)	Functional group
1.	3628.10	O-H group
2.	1456.76	C=C group
3.	1558.48	C=N group
4.	773.00	C-H/C-Cl

Table 4.7: IR absorption frequencies of functional groups in compound 01.

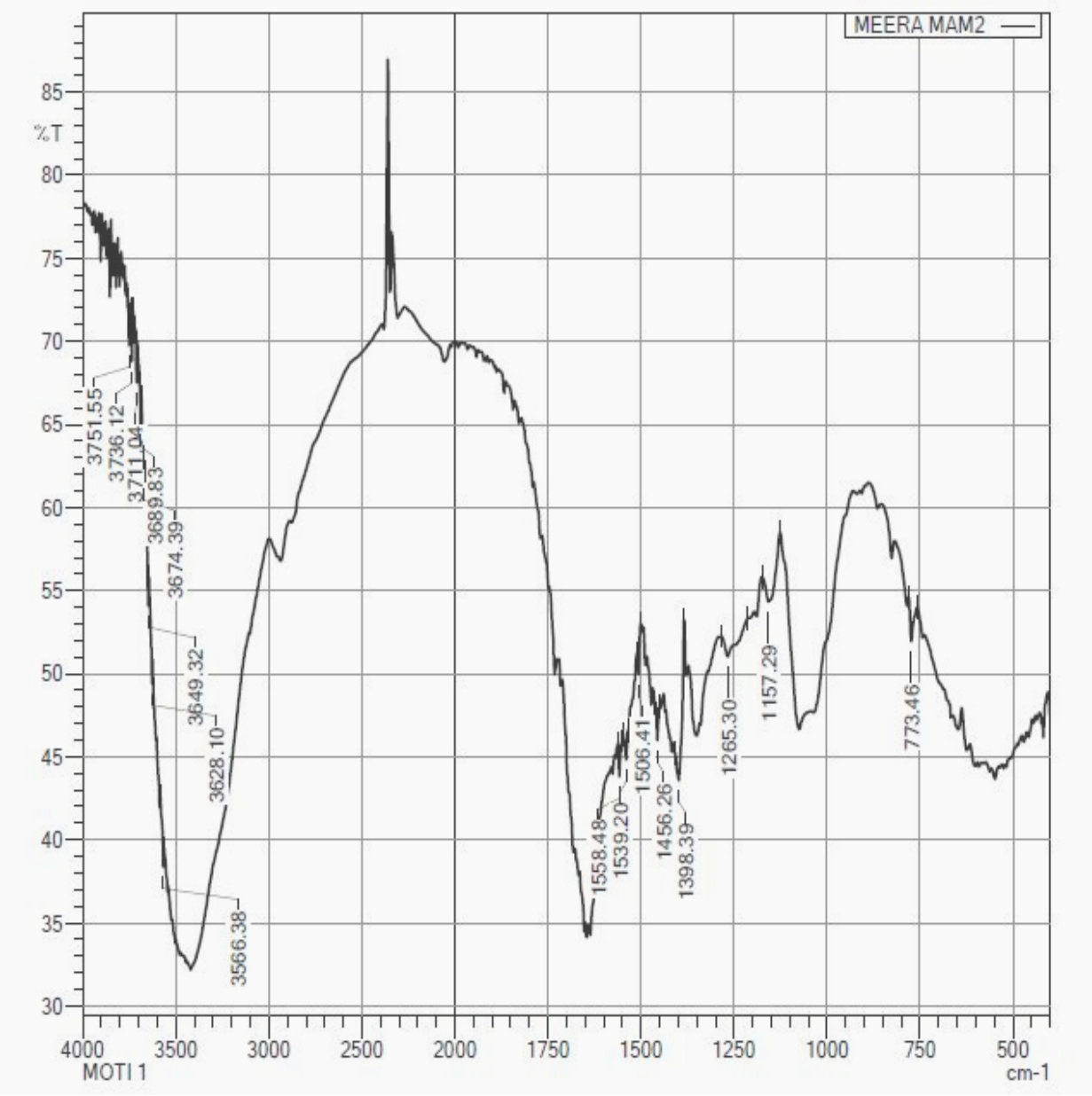

Figure 4.19: FTIR spectrum of the isolated compound 01.

4.7.4 λ max analysis through UV spectrophotometer of compound 01: fraction of endophyte bacterial bioactive metabolites (compound 01) was examined λ max by UV spectroscopy as shown in the figure 4.20. Fraction 01 has a λ max of 274.9 and 207.8 nm and Absorbance is 0.222. This result indicates a single bioactive metabolism as well as methanol fraction.

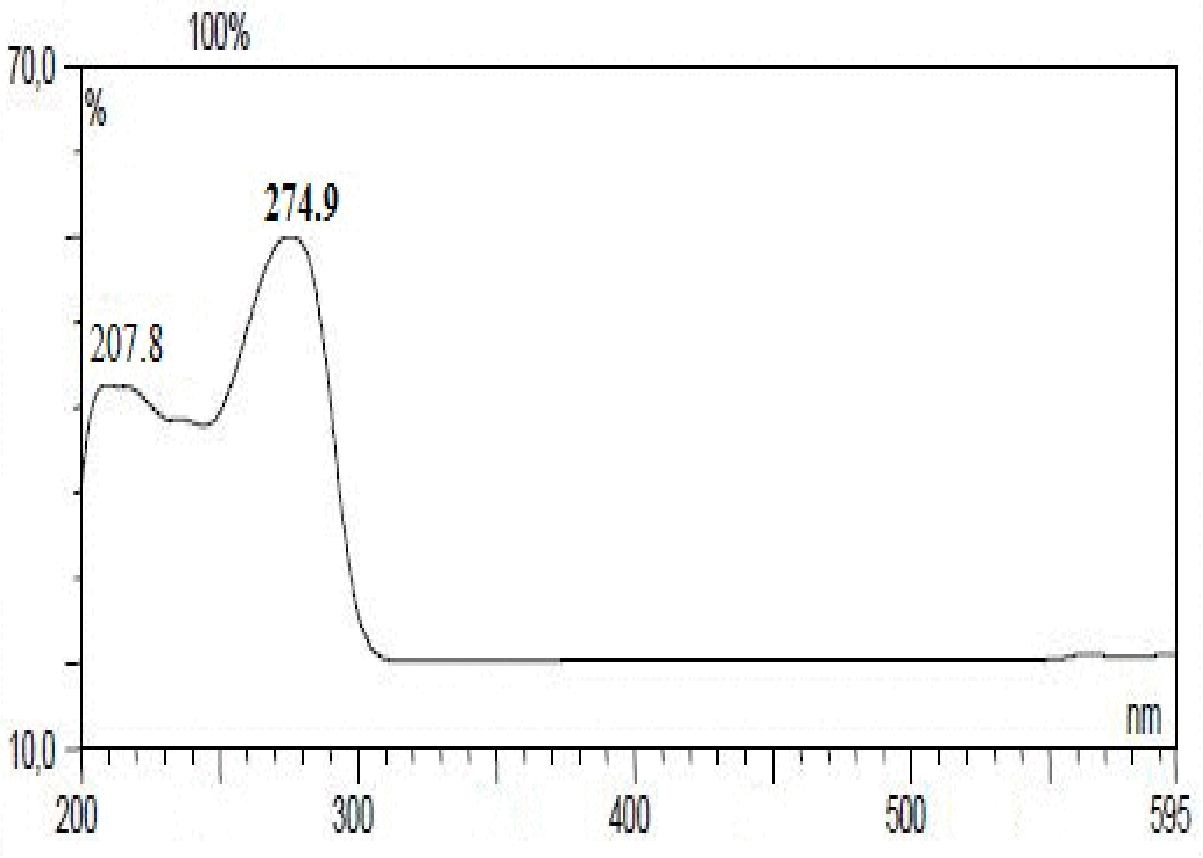

Figure 4.20: λ max analysis of compound 01 from isolated endophyte bacteria.

4.7.5 HPLC spectrum analysis of fraction 01: In this bio-instrument, When the sample was run in a circular analytical cartridge (C18) at 280 nm for 10 minutes, the absorption peaks were shown in Figure 4.21. Antimicrobial metabolite 01 indicated the absorption peaks at RT (Retention time in min) 1.894, 2.429, 2.900 and 3.210. the Major peaks were obtained at retention time (2.900 and 3.210). All these different peaks indicate the presence of bioactive metabolites.

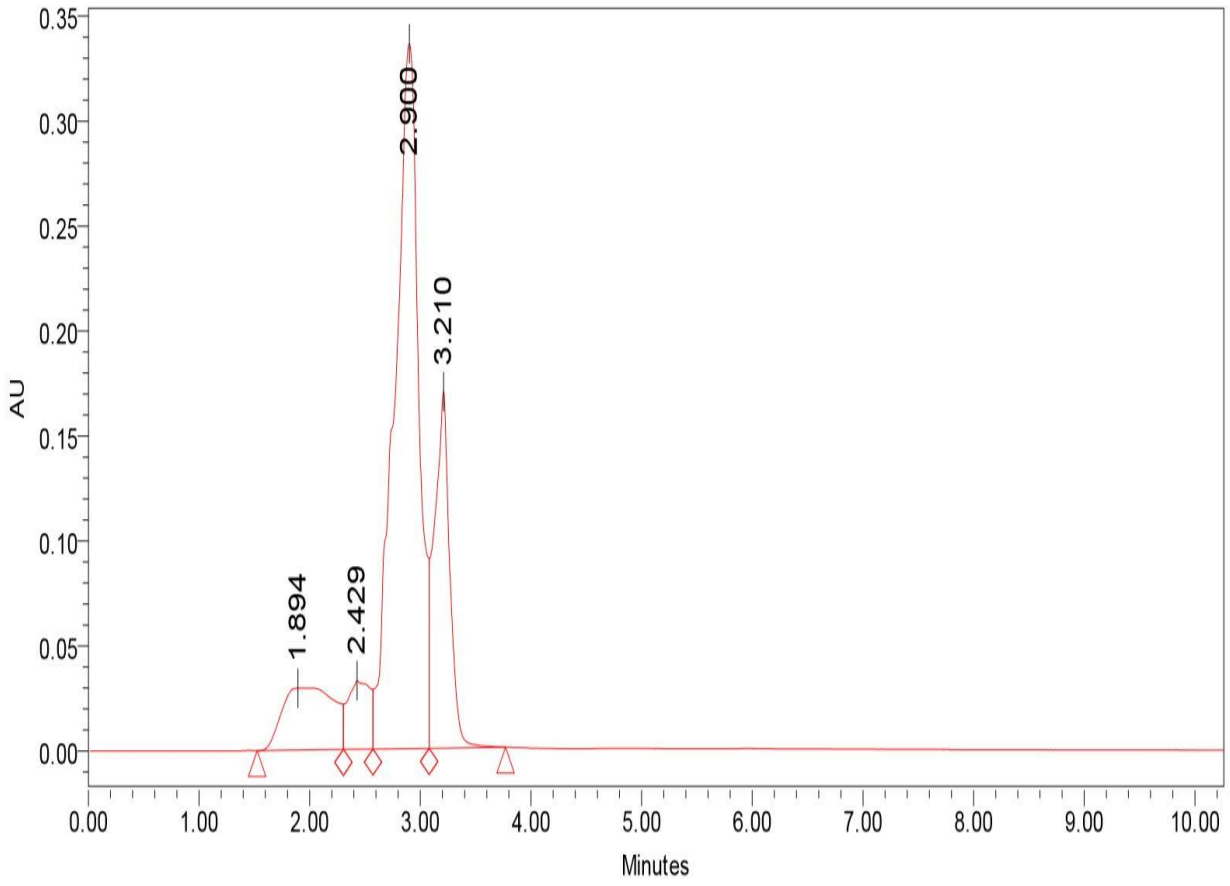

Figure 4.21: HPLC analysis of the compound 01.

4.7.6 Elucidation of the structure of the isolated fraction 01: After a detailed study of all the above spectrum (UV, HPLC, IR, MS, and NMR) found an Expected structure of the isolated compound 01, the approximate structure of compound 01 is such that it represents a possible linear structure that follows, $C_{40}H_{72}N_2O_{10}$. And the composition of compound 01 is as follows, C (59.55%), H (12.72%), N (6.08%), and O (21.65%).

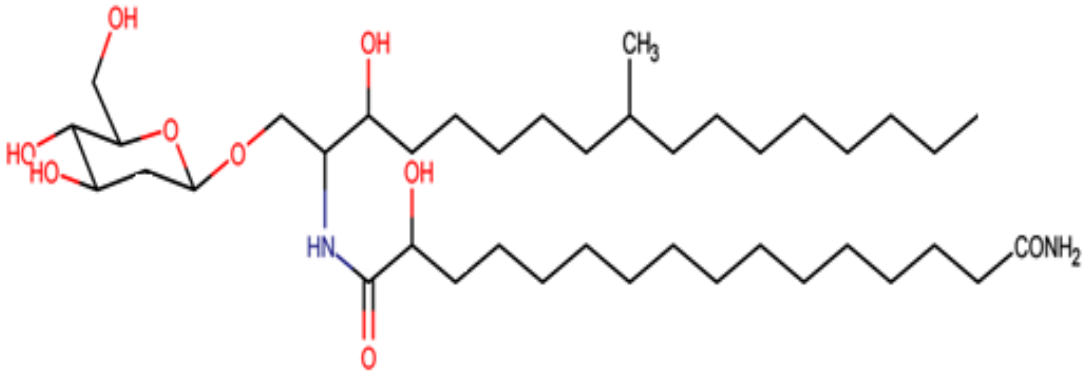

Figure 4.22: expected structure and IUPSC name of compound 01: (E)-N1-((E)-18-amino-3-hydroxy-9-methyl-18-oxo-1-(((2R, 3R, 4S, 5S, 6R)-3, 4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yl) oxy) octadec-4-en- 2-yl)-2-Hydroxyhexadec-3-enediamide

Compound 01 was checked anti-properties against various bacteria and fungi. The bacteria and fungi on which the anti-microbial properties were tested are as follows, *X. oryzae*, *E. coli*, *Streptococcus pneumonia*, *B. subtilis*, *Staphylococcus hyicus*, *B. sphaericus*, *Staphylococcus aureus*, *pseudomonas aeruginosa* and *Candida albicans*.

4.8.1 ^{1}H and ^{13}C of Compound 02: shows the ^{1}H-NMR spectra showed one alkane group attached to a primary carbon atom by exhibiting a singlet at δ 0.805 and two other carbonyl groups exhibiting an overlapped doublet (d). Two protons under the oxygen residue, showing a signal at δ 3.42, located at C-3 and C-17. The existence of an alcohol group was revealed by the broad signal at δ3.42. The rest of the signal between δ 4.08and 4.23 are due to phenol protons.

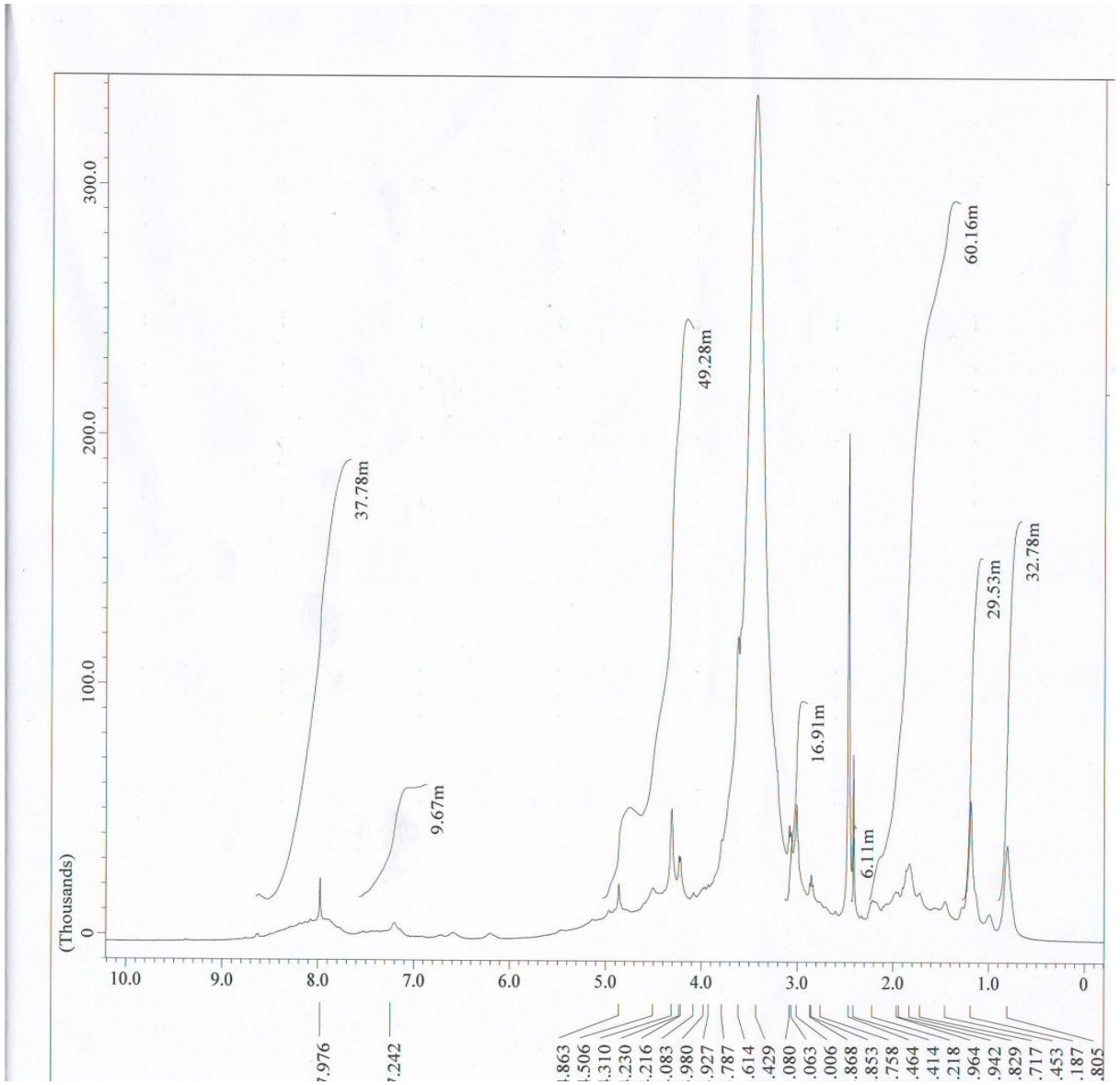

Figure 4.23: ^{1}H NMR spectrum of the isolated compound 02.

When ^{13}C NMR was performed for the second compound, various peaks were Found. which are as follows, **^{13}C**-NMR spectra confirm the presence of the three-alcohol group (C-OH) carbons by exhibiting signals at λ 70.76 ppm to 77.22 ppm and It is also seen in this graph that the two **^{13}C** signals are λ 97.38 ppm and λ 172.72 ppm respectively indicating alkyne (C≡C) and carboxylic (C=O), another signal at λ 21.70 ppm and λ 39.44 ppm is indicated to the two groups of alkanes (C-CH_3). respectively Two aldehydes (C-C=O) peaks between λ 40.11ppm to 40.45ppm were obtained.

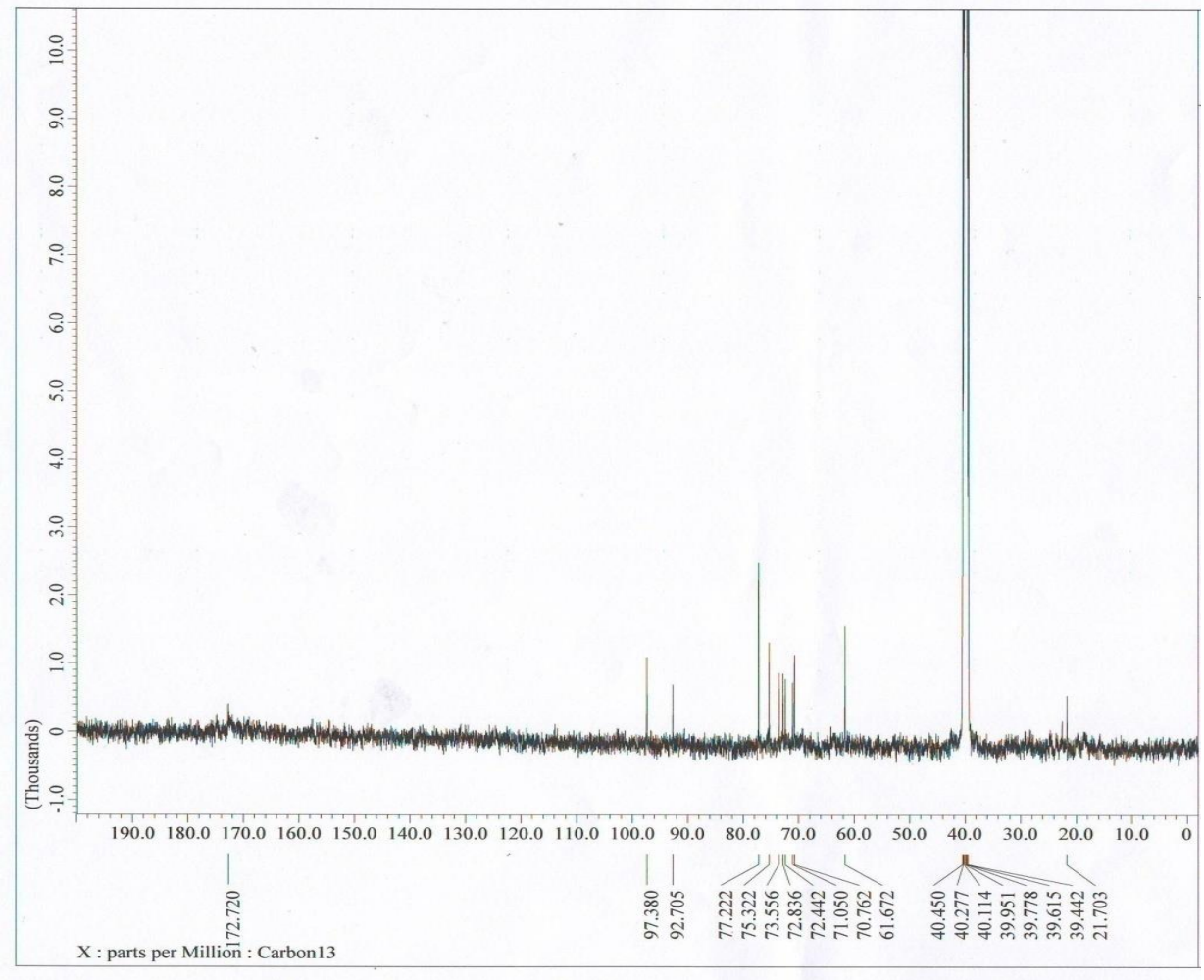

Figure 4.24: **^{13}**C NMR spectrum of the isolated compound 02.

4.8.2 Mass Spectrum analysis of compound 02: spectroscopy was performed for the second compound, some special molecular weight of this compound was observed, Due to the gradual loss of ESI-MS m/z 418.7, 432.6, 437.8 and 409.8 grams/mole units, these are fragments that confirm the presence of stretched chains of bioactive metabolites. Out of all these picks, the 418.7 peak indicates compound 2 as well as its molecular weight, which is the highest in the concentration (figure 4.25).

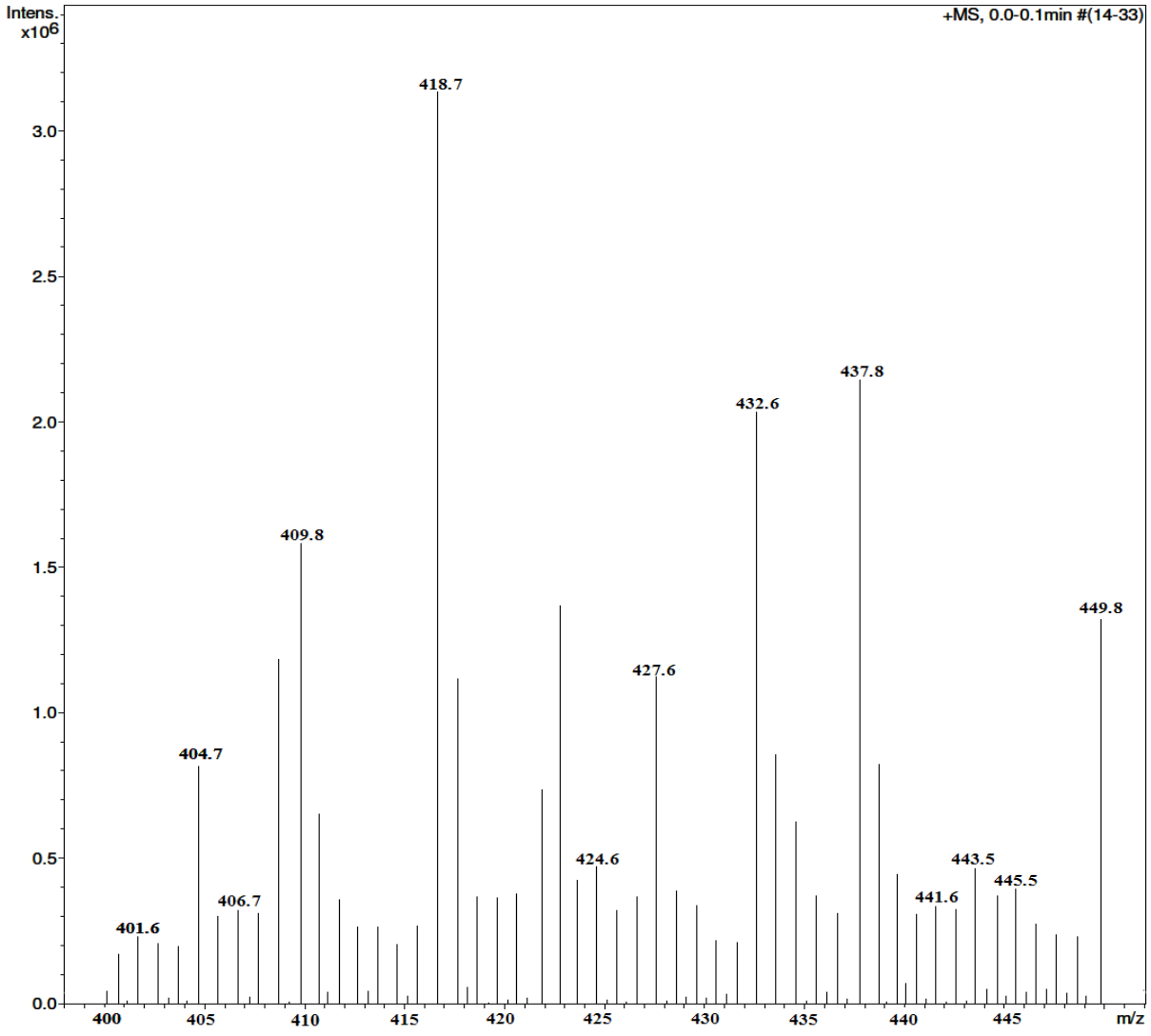

Figure 4.25: Mass spectroscopy analysis of compound 02.

4.8.3 Fourier transform infrared (FT-IR) spectroscopy of fraction 02: For a molecular study of a bioactive metabolite, Fourier transform infrared spectrophotometer was performed. And based on the FTIR peak, the chemical/structural properties of this bioactive compound were determined and it also revealed the functional groups. The weak absorption band of IR (KBr) υmax 401.10 to 420.48 cm^{-1} shows the presence of chloride, in bioactive metabolite.

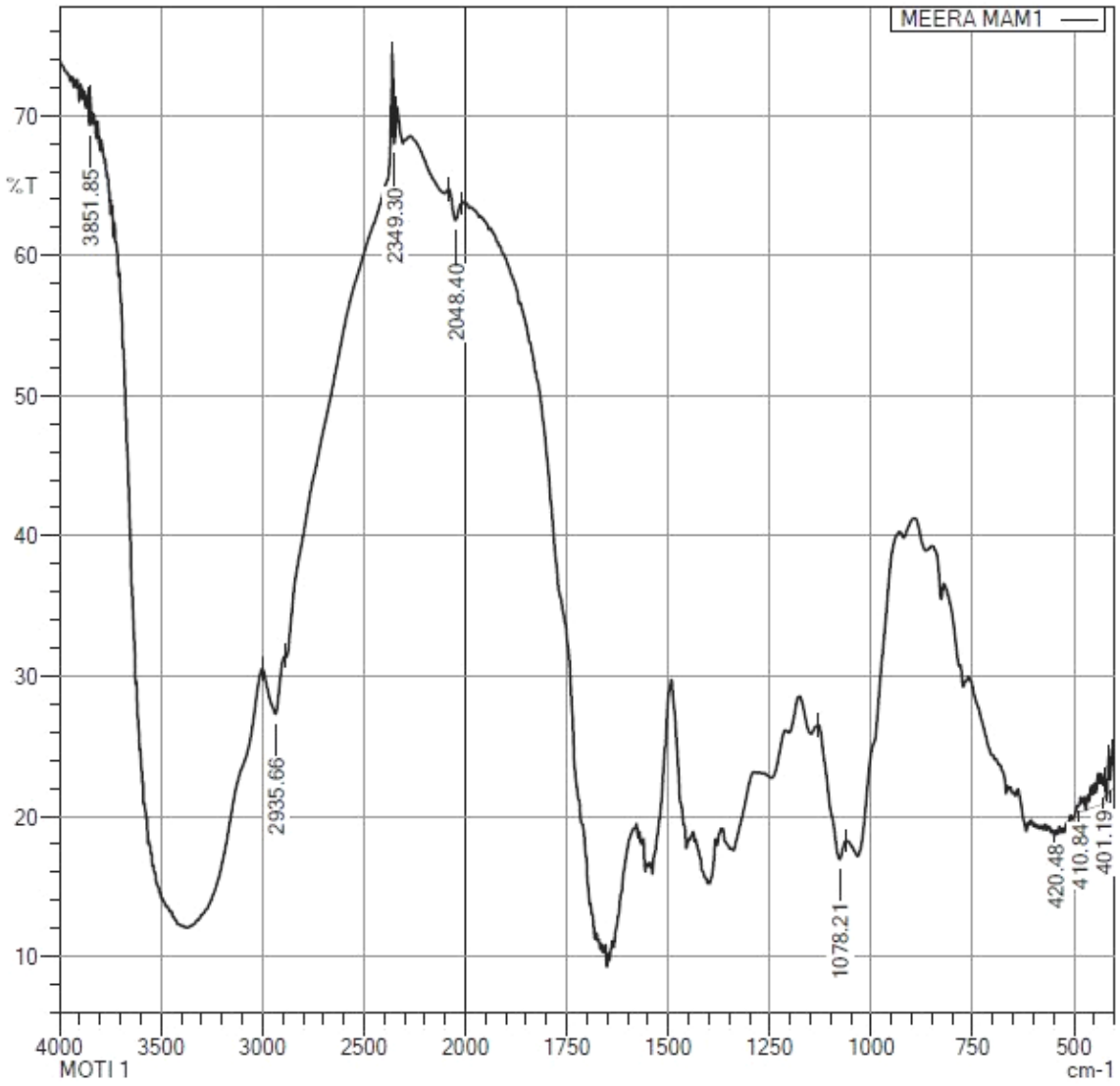

Figure 4.26: FTIR spectrum of the isolated compound 02.

The band at 1078.21 cm^{-1} is generally indicated to the alcoholic group. This O-H stretching frequency specify to the phenolic compound, this compound has maximum antimicrobial properties and, in this study, another peak was found at 2048.40 indicating that this peak is alkaline (C = C). As well as in this graph, another peak (3851.8 cm-1) appears which confirms the phenol group formation. Analysis of FTIR data is important in this study to detect different functional groups of these compounds. Based on all these different functional groups, the expected structure of a different compound can be developed. The FTIR data of the isolated compound from microbial bioactive metabolites are shown in the figure 4.26.

S. No	Characteristic Absorption (cm-1)	Functional group
1.	401.19 to 420.48	C-Cl group
2.	1078.21	C-OH group
3.	2048.40	C=C group
4.	2935.66	C-H group

Table 4.8: IR absorption frequencies of functional groups in compound 02.

4.8.4 λ max analysis through UV spectrophotometer of compound 02: The UV-spectrophotometer was used to detect the maximum absorption of the bioactive compound 02. The UV absorption spectrum of bioactive compound 02 was found to be a maximum of 206 and 273.5. The UV spectral data of the second fractionated isolates showed the maximum absorbance peaks in the wavelength range of 206-273.5 nm may be an indicator for the presence of antimicrobial compounds in the bacterial culture extracts. Furthermore, the pointed spectral data are compatible with Saadoun et al., 1999.

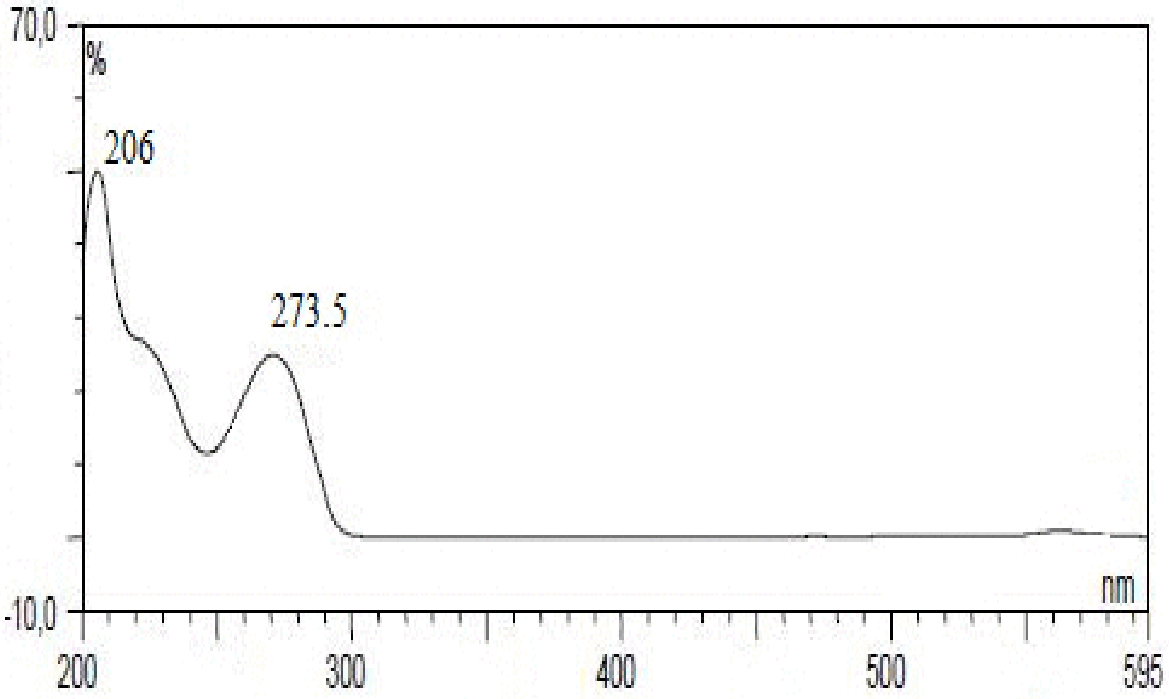

Figure 4.27: λ max analysis of isolated compound 02.

Therefore, these strains (EFB-03) formed a broad-spectrum of antimicrobial bioactive compounds or numerous compounds with different activities. The spectral data are consistent with those obtained by Slavic et al., 2005; Swaadoun et al., 1999.

4.8.5 HPLC spectrum analysis of compound 02: Despite the various facts that, HPLC results of antimicrobial agents at conditions of pressure, 71.96% with a flow rate of 1.5 and pH 7.2, gave a peak at 0.389, 3.867 and 4.300 retention time (minutes).

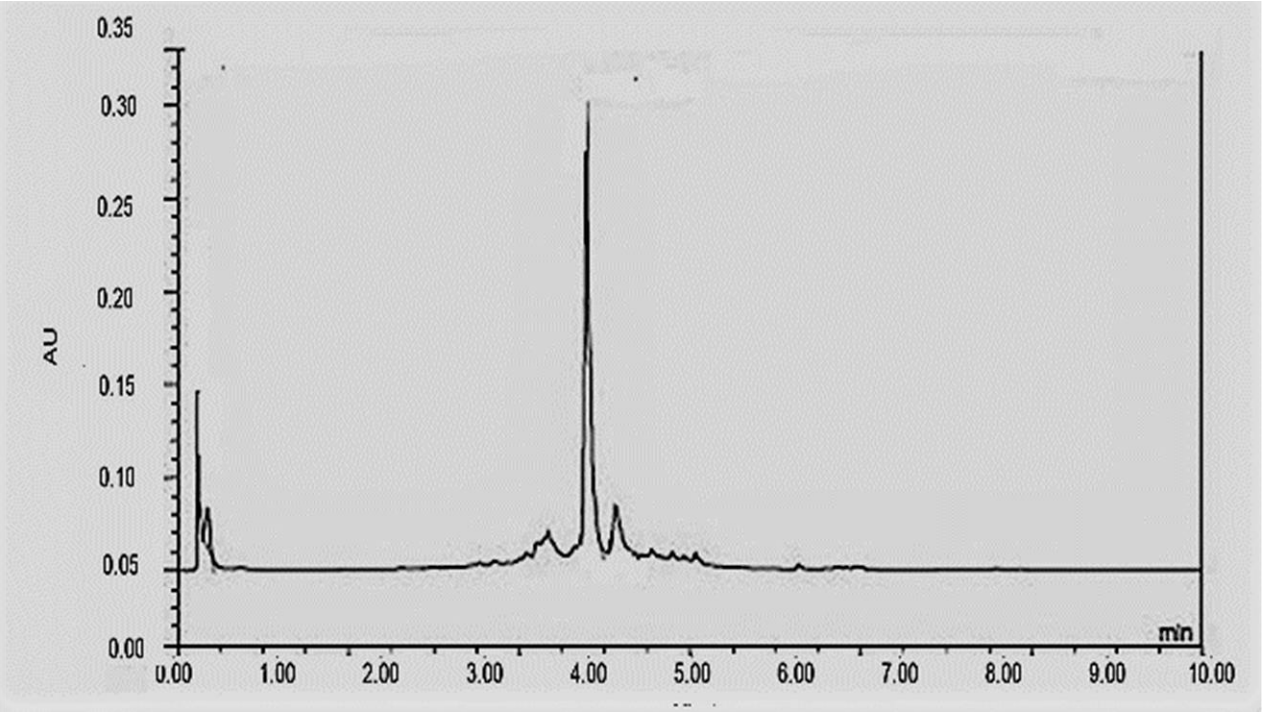

Figure 4.28: HPLC analysis of the compound 02.

Which indicates that this peak is one of the bioactive metabolites. The HPLC analysis of compound 02 (Figure 4.28), we got 03 clear peaks of which 3.867 Peak is the peak of the main bioactive metabolites.

Compound 02 was found active against- *X. oryzae, E. coli, Streptococcus pneumonia, B. subtilis, Staphylococcus hyicus, B. sphaericus, Staphylococcus aureus, and pseudomonas aeruginosa* and *Candida albicans.*

4.8.6 Elucidation of the structure of the isolated compound 02: After analyzing all the data, found an expected structure of compound 02 which is of this type (figure 4.29) compound 02 is as follows the probable linear structure is $C_{24}H_{49}N_3O_9$. And the compositions of compound 02 are C (55.05%) H (9.43%) N (8.02%) O (27.50%) and ESI-MS (m/z) 418.7.

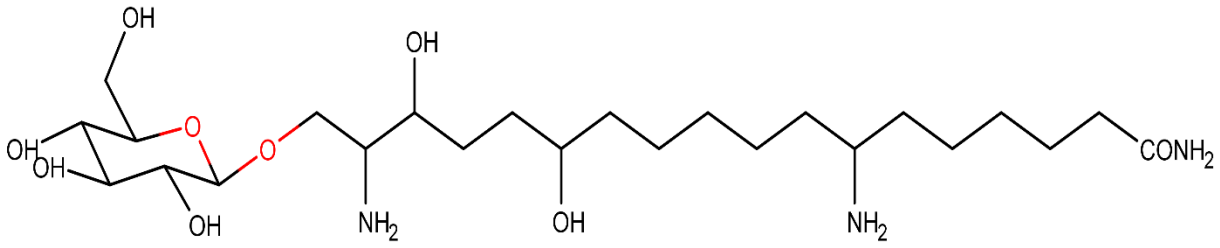

Figure 4.29: expected structure and IUPSC name of compound 02: 7,17-diamino-13,16-dihydroxy-18-(((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6 (hydroxymethyl)tetrahydro-2H-pyran-2-yl) oxy) octadecanamide.

4.9.1 ^{1}H and ^{13}C NMR of Compound 03: According to this 1H NMR (DMSO-D6, 300 MHz), several peaks can be seen in this graph (Figure 4.30). A peak with a range of 0.93 to 0.95 indicating the existence of alkanes (C-C-H) and attached to a primary carbon atom, there is an indication of another peak here, whose value is 1.26 to 1.37 is indicating to alcohol (C-O-H). The 03 protons under oxygen residue is giving a signal at δ 3.41 which is located on C-6, C-10 and C-13.

This compound was dark brown in color, the composition of these compound is as follows: C (67.98%) H (10.73%) N (1.89%) O (19.40%) molecular formula $C_{22}H_{42}N_4O_7$, ESI-MS (m/z)

516.6. ^{1}H NMR (dmso-d6, 300 MHZ), 3.36-3.76ppm (m,2H-glucose), 4.21 ppm (m,2H), 4.52 ppm (m,1H), 1.96 (t,4H), 1.77(t,2H), 1.29 (m,10H), 1.20 (m,12H), 0.95 (d,3H),0.88 (m,6H), 1.37 (m,4H).

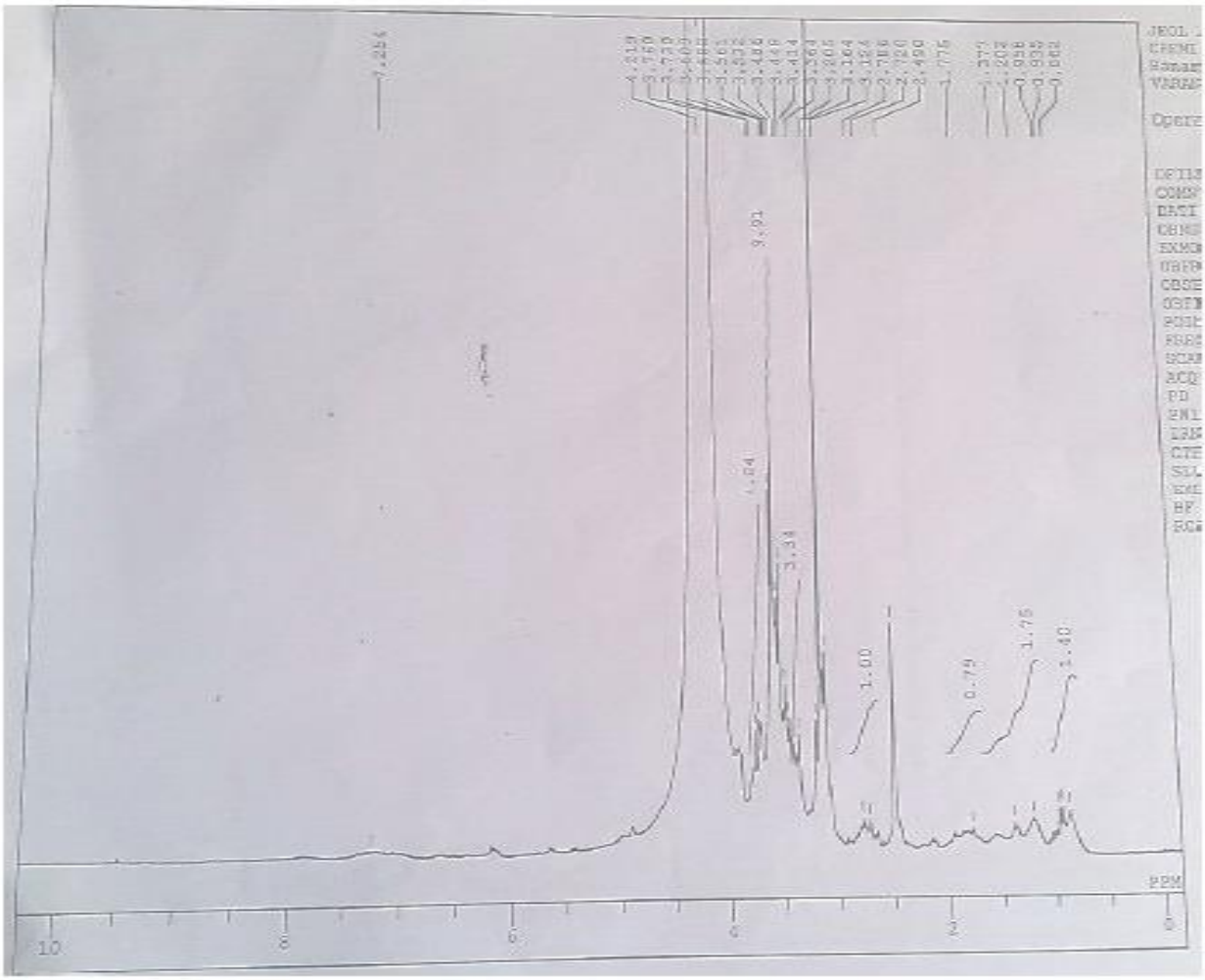

Figure 4.30: ^{1}H NMR spectrum of the isolated compound 03.

When ^{13}C NMR was done for the third compound, the peaks of different values were found which are as follows. ^{13}C NMR (75 MHZ) DMSO-d6;182-188 ppm (-C=O), 40.06 ppm (aldehydes group), 47.04 ppm-63.31 (C-N), 27-38 ppm (C-C), 69.64 ppm- 77.32 (C-OH), 75ppm (C-O-C), 88.63-97.95 pppm (C≡C). And in this graph, there are two such peaks (188.45ppm-194.58 ppm)

which are confirming the existence of aldehydes (C=O) group. And there are some peaks (118.14ppm- 129.34 ppm) that are confirming the aromatic compound to be present and two another peak 39.50 ppm-39.7 ppm responsible for the carboxylic acid (C-C≡O).

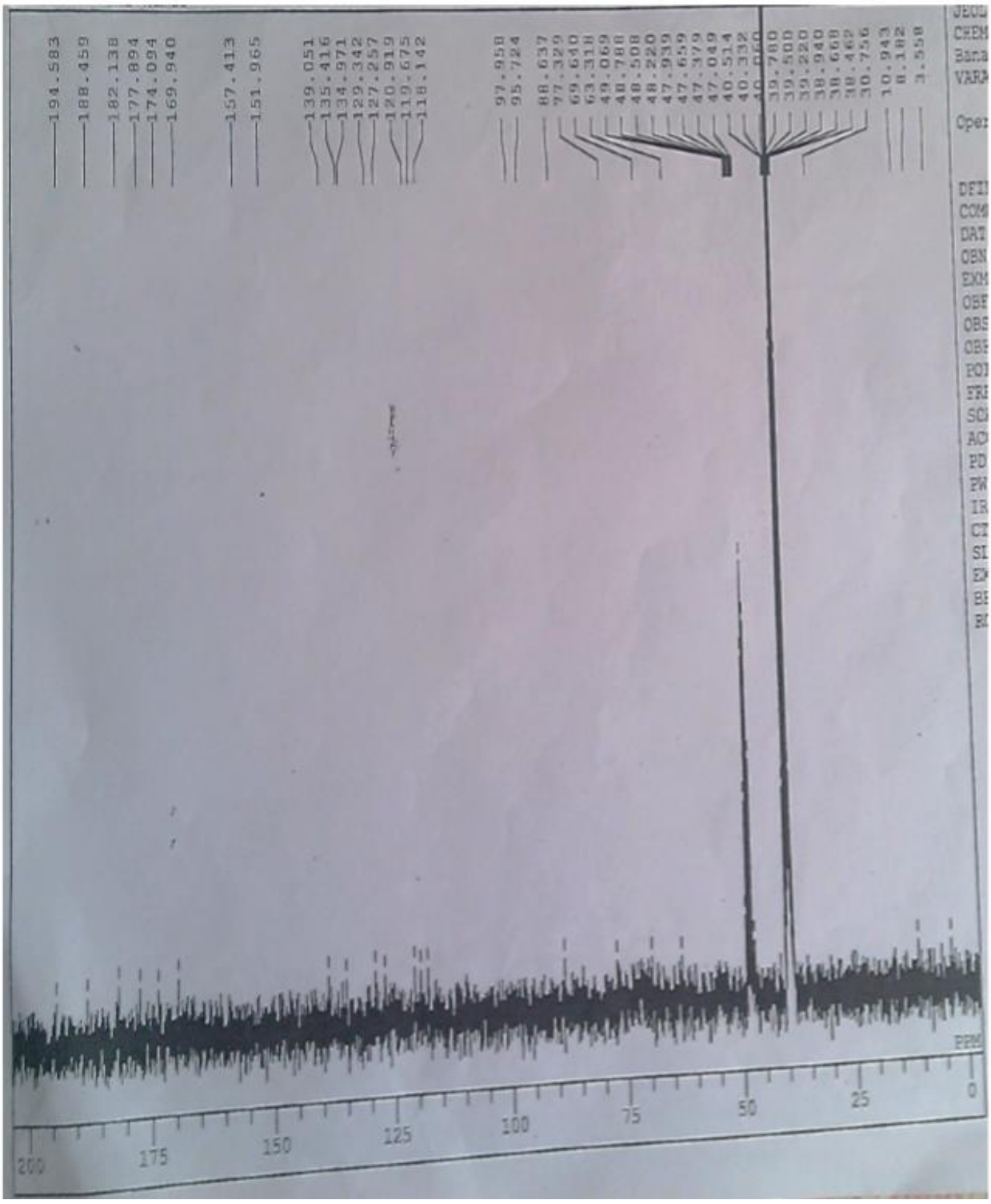

Figure 4.31: ^{13}C NMR spectrum of the isolated compound 03.

4.9.2 Mass Spectrum analysis of compound 03: Peaks of different molecular weights were found when compound 03 was performed by mass spectrophotometer. which is from ESI-MS m/z 516.6, 509.8 and 532.6 grams / mole are the fragments due to sequential loss of units which confirms the existence of stretched chains of bioactive metabolites. Out of all these picks, the 516.6 peak indicates compound 3, which is the highest in the concentration. That is molecular weight of compound 03 is 516.6.

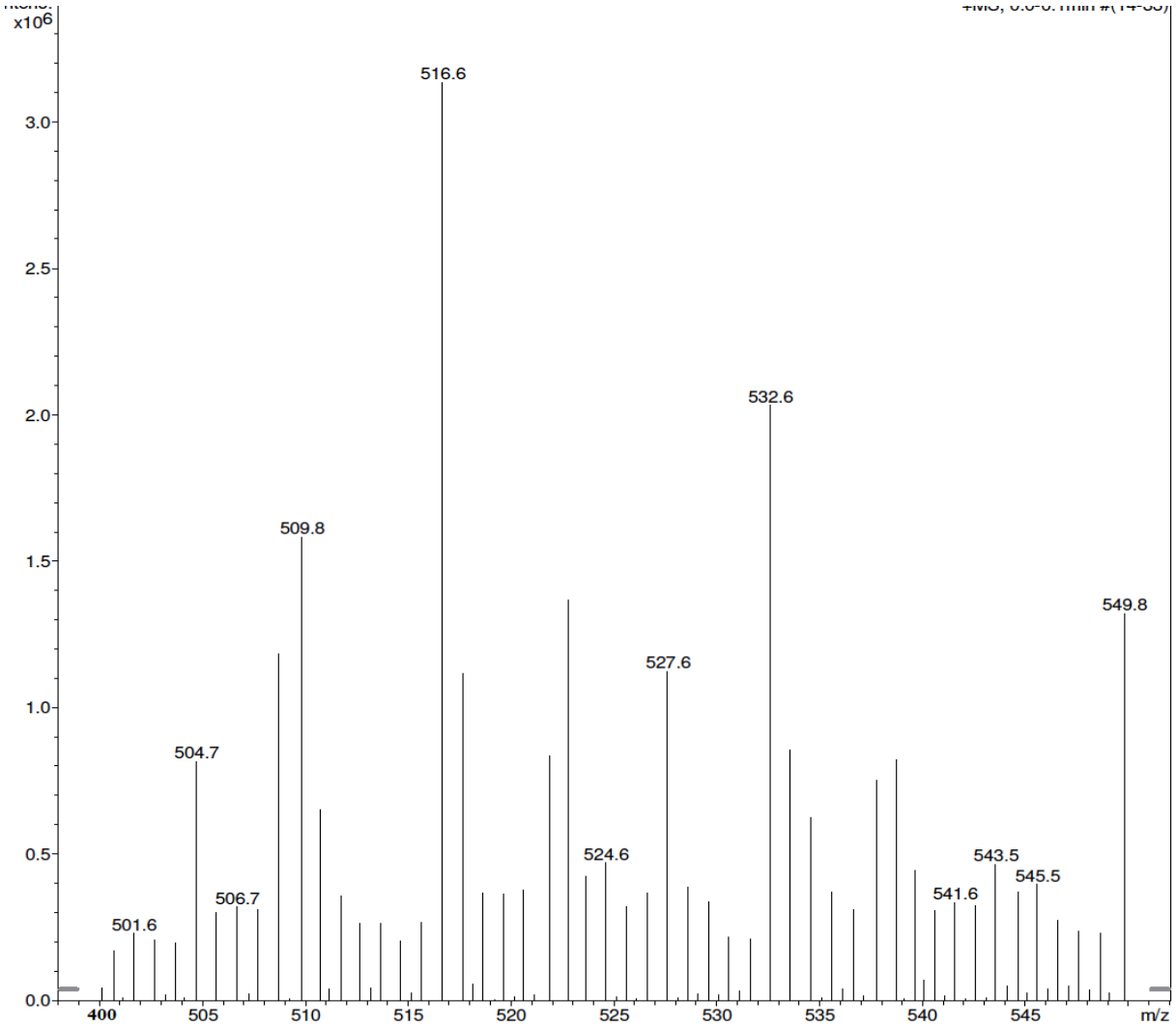

Figure 4.32: Mass spectroscopy analysis of compound 03.

4.9.3 Fourier transform infrared (FT-IR) spectroscopy of compound 03: The Fourier transform infrared spectrophotometry (FTIR) was performed to detect functional groups of compounds 03. After performing the Fourier transform infrared spectrophotometer of compound 03, various functional groups were observed. which is like this. The weak absorption band of IR (KBr) υmax 560.67 to 630.89 cm^{-1} shows the presence of C-Cl, in the bioactive metabolite.

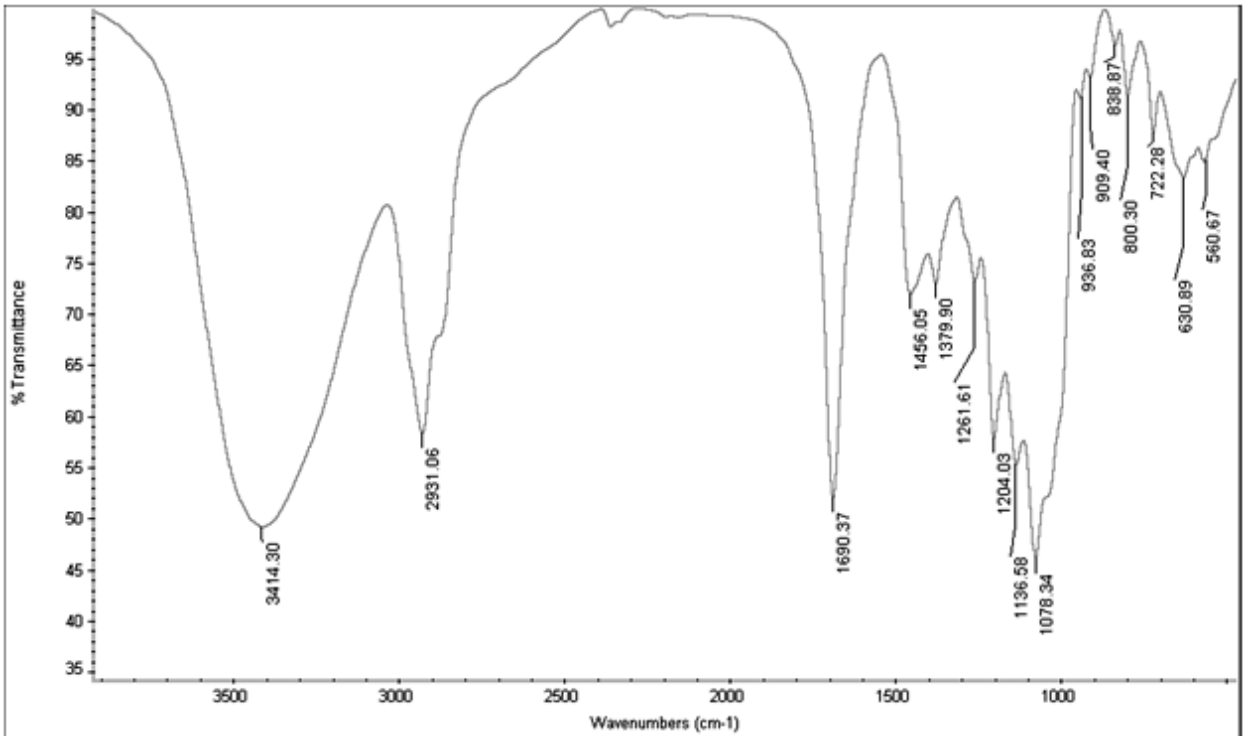

Figure 4.33: FTIR spectrum of the isolated compound 03.

Another peak was obtained on 3414 cm^{-1} indicating that this peak is of alcohol. Some other different peaks were also seen, such as 1890, 1857, and 1379 cm^{-1} which orderly confirms the existence of amide, aromatic compound and Nitro-compounds (NO_2).

S. No	Characteristic Absorption (cm-1)	Functional group
1.	3414.00	C-OH group
2.	1690.37	C=O group
3.	1456.05	C=C group
4.	1379.90	NO_2 group

Table 4.9: IR absorption frequencies of functional groups in compound 03.

4.9.4 λ max analysis through UV spectrophotometer of compound 03: the compound 03 was investigated through UV spectroscopy, whose image is shown in figure number 4.34 given below.

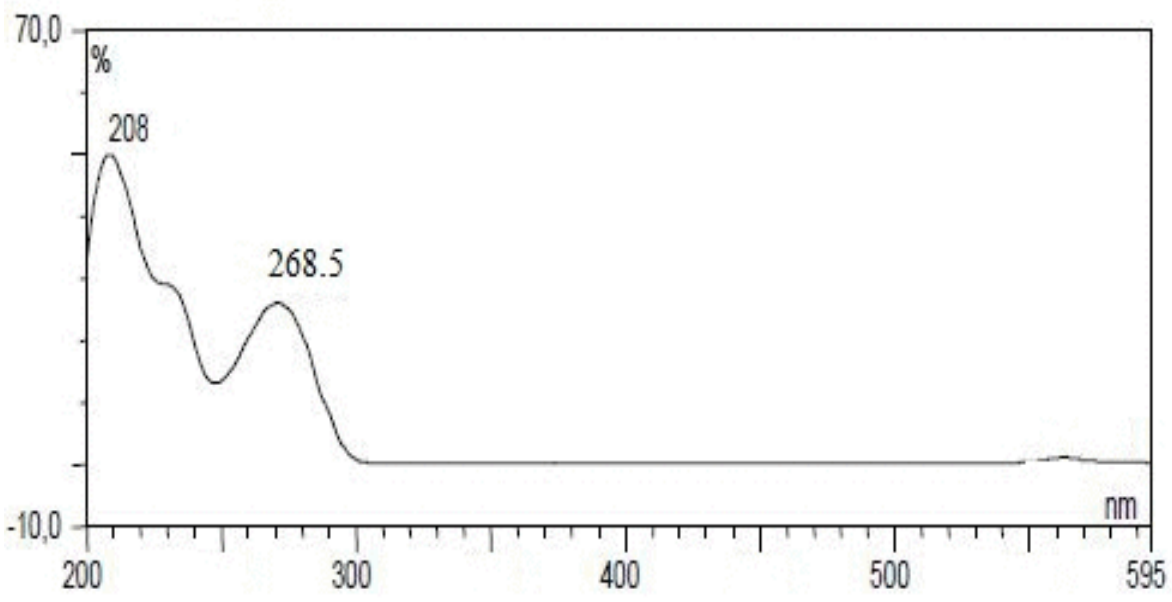

Figure 4.34: λ max analysis of isolated compound 03.

In the third compound, at maximum absorption of 0301, the value of λ max is 208 nm. (The 208 nm wavelength is inducing 50% transmittance, and when the transmittance was changed to observance, the maximum absorption 0.301 was found). This result indicated that compound 03 is formed only through a single type of molecule.

4.9.5 HPLC spectrum analysis of compound 03: HPLC is being routinely used for the analytical assessment of various bioactive metabolites. In the current study, HPLC outline of the antimicrobial compounds 03 was performed by circular analytical cartridge (C18) at 280 nm for 10 minutes, the absorption peaks were showed in Figure 4.35. Antimicrobial compounds 03 indicated absorption peaks with Rf (retention time in minutes) 3.50 and 6.21. Major peaks of this analysis are 3.500. The major peak of this HPLC indicates a bioactive metabolite compound.

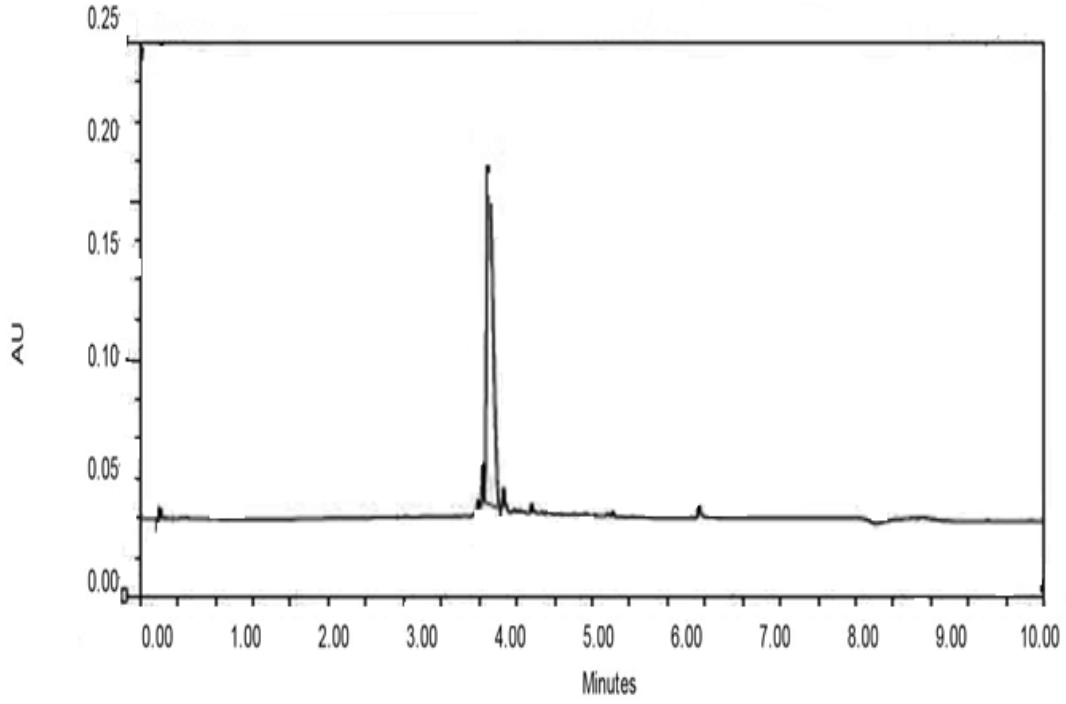

Figure 4.35: HPLC analysis of the compound 03.

4.9.6 Elucidation of the structure of the isolated compound 03: considering all the data, an expected structure of Compound 03 is drawn and its linear structure is $C_{22}H_{42}N_4O_7$. And the compositions of compound 03 are C (10.73) H (67.98%) N (1.89%) O (19.40%) and ESI-MS (m/z) 516.6

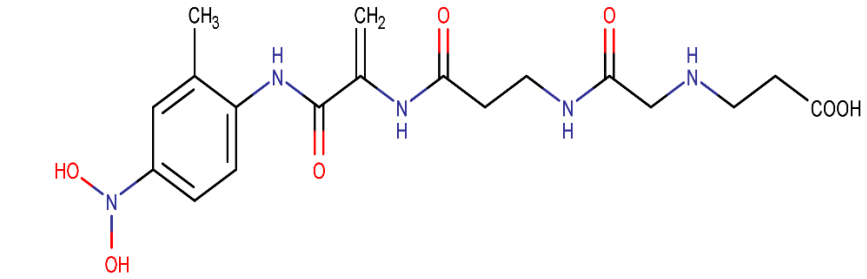

Figure 4.36: expected Structure of compound 03.

Compound 03 was observed active against-*E. coli, Streptococcus pneumonia, B. subtilis, Staphylococcus hyicus, B. sphaericus, Staphylococcus aureus, and pseudomonas aeruginosa, Candida albicans, Sclerotium rolfsii, Sclerotinia, scleratiourm and Penicillin sp.*